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STUDY ON ASSOCIATION OF ATP-BINDING CASSETTE TRANSPORTER A1 (ABCA1) GENE POLYMORPHISMS IN TYPE 2 DIABETES MELLITUS AMONG NORTH INDIAN POPULATION

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ABBREVIATIONS

Sn. No.	Abbreviation	Full form
1.	**ABCA1**	ATP binding cassette transporter A 1
2.	**ADP**	Adenosine diphosphate
3.	**Akt**	Protein kinase B
4.	**apo -A1**	Apolipoprotein A1
5.	**ATP**	Adenosine triphosphate
6.	**BMI**	Body mass index
7.	**CETP**	Cholesterol ester transfer protein
8.	**DCCT**	Diabetes control and complications trial DM – Diabetes mellitus
9.	**EDTA**	Ethylene diamine tetra acetic acid
10.	**FFA**	Free fatty acids
11.	**FPG**	Fasting plasma glucose
12.	**HbA1c**	Glycosylated hemoglobin
13.	**HDL-c**	High density lipoprotein cholesterol
14.	**HSL**	Hormone sensitive lipase
15.	**IFG**	Impaired fasting glucose
16.	**IGT**	Impaired glucose tolerance
17.	**LDL-c**	Low density lipoprotein cholesterol
18.	**LPL**	Lipoprotein lipase
19.	**mTORC1**	Mammalian target of rapamycin complex 1
20.	**NAD**	Nicotinamide adenine dinucleotide
21.	**NCD**	Non communicable disease
22.	**NCEP ATP**	National Cholesterol Education Programmed Adult Treatment Panel
23.	**NEB**	New England Biolabs
24.	**NEFA**	Non esterified fatty acids
25.	**NGSP**	National Glycohemoglobin Standardization Program
26.	**PI3K**	Phosphoinositide 3- kinase
27.	**SOD**	Superoxide dismutase
28.	**TGL**	Triglycerides
29.	**VLDL-c**	Very low-density lipoprotein cholesterol
30.	**WHO**	World health organization

INDEX

S.NO.	CONTENT	PAGE NO.
1.	INTRODUCTION	**1**
2.	AIM AND OBJECTIVES	**4**
3.	REVIEW OF LITERATURE	**5**
4.	MATERIAL AND METHODS	**19**
5.	RESULTS	**33**
6.	DISCUSSION	**41**
7.	CONCLUSION	**47**
8.	SUMMERY	**50**
9.	BIBLIOGRAPHY	**51**
10.	ANNEXURES	**61**

INTRODUCTION

Diabetes mellitus (DM) is a metabolic condition characterized by chronic inflammation and abnormal glucose, protein, and lipid metabolism, which is thought to be caused by a variety of factors. It is caused by either impaired insulin secretion, insulin action, or both (1). Based on this, two primary types of diabetes were identified: type 1 and type 2. Type 1 diabetes is caused by autoimmune or viral death of β cells, resulting in a lack or significant decrease in insulin output. This condition affects around 10% of the diabetic population. Type 2 diabetes is the most frequent kind, accounting for more than 90% of diabetic individuals (1).

Type 2 DM was previously classified as non-insulin dependent DM (2). Type 2 diabetes has a complicated etiology that includes genetic, environmental, and behavioural risk factors, and it has been identified as one of the four main noncommunicable diseases (NCDs) by the World Health Organization. Over the last two decades, type 2 diabetes has afflicted over 150 million individuals globally (1). Asia has emerged as a hotspot for early-onset type 2 diabetes, with India predicted to experience a significant increase over the next two decades. Type 2 diabetes affects around 62 million people in India. Type 2 diabetes is estimated to impact up to 79.4 million people in India by 2030 (3).

Complications of type 2 diabetes have become the main cause of death and morbidity worldwide. Cardiovascular disease is becoming more common among those with type 2 diabetes (4, 5). Even while epidemiological studies have revealed that HDL-cholesterol (HDL-c) levels are negatively related to the risk of coronary artery disease and its thrombotic consequences, it is becoming clear that HDL-c functioning is equally significant as HDL-c levels. This is because HDL-c not only promotes reverse cholesterol transport, but it also has anti-oxidative, anti-inflammatory, anti-thrombotic, and vasodilating properties (6).

Previous studies showed that genetic and environmental factors play an important role in influencing beta-cell activity and shape, and therefore, tissue insensitivity to insulin in type 2 DM. The primary

causes of the illness include physical inactivity, a sedentary lifestyle, smoking, an unhealthy diet, being overweight, and obesity (7).

In addition to harmful mutations, genetics is a key factor in influencing the risk of type 2 DM. When compared to those without a family history, the risk in the first-degree relative of a person with T2DM is almost three times higher. To date, more than 50 potential genes have been found to be involved in the global predisposition to type 2 DM. This illness develops as a result of harmful mutations in these genes (8, 9). One of the most common genes in type 2 diabetes is adenosine binding cassette transporter 1 (ABCA1), a crucial gene that encodes the ATP-dependent medication efflux pump. ABCA1 transporter facilitates transportation of lipids by using an ATPase-dependent mechanism (10). Defects in the ABCA1 gene impair the efflux of cholesterol and phospholipids to lipid-poor apolipoproteins (apo-A1 and apo E), which causes cholesterol to build up in the cells and causes type 2 DM and cardiovascular disease (11).

Study of genetic variations in ABCA1 gene is an approach to the investigation of type 2 DM. Single Nucleotide Polymorphism (SNPs) which are generally found throughout the genomic DNA, are the most prevalent type of genetic variations (12). A large number of SNPs in the susceptibility genes have been identified in developing T2DM. These polymorphisms nearly always affect insulin action, glucose metabolism, pancreatic beta-cell function, and other metabolic conditions including energy intake and lipid metabolism (13). SNPs within the *ABCA1* gene may play a direct or indirect role in the function of protein associated with the development of T2DM. A number of these loci are found in non-coding areas of genes, such as the promoter, splice site, and introns, or coding exon regions, where they may be synonymous or nonsynonymous mutations. The loci found in the ABCA1 gene or in a regulatory region, surrounding this gene may have a more direct impact on type 2 DM by influencing gene function. The structure or functionality of the ABCA1 protein may be marginally impacted by each SNP. However, numerous SNPs can have a role in the development of type 2 diabetes, and this impact is community specific. SNPs with no biological or clinical effects can be

employed as markers to map and identify disease-causing mutations in ABCA1 or other associated genes, allowing for early identification and treatment of T2DM and lowering disease-related mortality (14, 15). The processes by which identified SNPs interact and contribute to T2DM are not completely understood. In this work, we investigated the function of SNPs in ABCA1 gene polymorphisms in T2DM and gave insights into their possible contribution to disease progression. For this study, we selected R219K (rs2230806) and C69T (rs1800977) SNPs of ABCA 1 gene to understand a more precise assessment of the role of these SNPs in T2DM.

AIM & OBJECTIVES

Aim: To determine the genetic polymorphisms of R219K (rs2230806) and C69T (rs1800977) of ABCA1 gene in type 2 DM.

Objectives:

1. To study the cholesterol (total, triglycerides, HDL and LDL) in type 2 DM patients and non-diabetic healthy individuals.

2. To study the genotype and allele frequencies of ABCA1 gene polymorphism (R219K and C69T**)** in type 2 DM patients and non-diabetic healthy individuals.

3. To find out the association between ABCA1 gene polymorphism and type 2 DM.

REVIEW OF LITERATURE

DM is the foremost non-communicable disease which is prevalent worldwide. It is a complex metabolic disorder characterized by chronic hyperglycemia, related with alterations in metabolism of carbohydrate, protein and fat, primarily due to defective insulin secretion or action or both (8).

Epidemiology of type 2 DM:

The disease burden associated to type 2 DM is rising globally, fuelled by the comprehensive rise in the prevalence of obesity and detrimental lifestyles. The latest estimates show a worldwide prevalence of 382 million people with diabetes in 2013, anticipated to rise to 592 million by 2035 (9). Caribbean region and North America has got the maximum prevalence of disease (11%), subsequently the Middle East and North Africa have a prevalence of 9.2%(10). It was estimated that almost 1 million Indians expire due to type 2 DM annually with the common age of onset being 42.5 years (11, 12). When compared to urban population, prevalence of type 2 DM is just one-fourth in the rural areas (13,14). Type 2 DM has a male preponderance in early middle age when compared to women (15). In developing countries, people with diabetes are usually in 45 to 64 years of age while in developed countries majority of them are greater than 64 years of age (16).

Criteria for diagnosing type 2 DM (17):

- Glycosylated hemoglobin (HbA1c) ≥ 6.5% or
- Fasting Plasma Glucose (FPG) ≥ 126 mg/dL or
- 2-hour plasma Post prandial glucose ≥ 200mg/dL during an Oral Glucose Tolerance Test or
- Random plasma Glucose ≥ 200 mg/dL with signs and symptoms of hyperglycemia.

Type 2 DM:

The foremost type of DM is type 2. It affects more than 90% of the inhabitants suffering from DM worldwide. There is a swift increase in the number of type 2 DM patients and this sizzling growth is noted in both rural and urban areas. It is characterized by disproportionate hepatic glucose production, erratic level of resistance to insulin action, decreased insulin secretion, and abnormalities in fat metabolism.

Risk factors for type 2 DM (17):

Sedentary lifestyle, diet and associated epidemiological conversion has been recognized as risk factors for type 2 DM. Other major risk factors are listed below.

- Family history of DM
- Overweight with body mass index(BMI) ≥ 25 kg/m^2
- Lack of physical activity
- Ethnicity
- History of impaired fasting glucose(IFG)/impaired glucose tolerance (IGT)
- Blood pressure $\geq$ 140/90 mmHg
- Triglyceride(TGL) levels ≥ 250 mg/dL
- HDL-c ≤ 35 mg/dL
- Gestational DM
- Polycystic ovary syndrome (PCOS)

Pathogenesis of type 2 DM:

Type 2 DM is found to be as a result of combined effect of both genetic and acquired etiologies. This establishes a negative influence on the beta cell function and also on the sensitivity of the tissues to insulin (18). For more than a few years, it was speculated

whether the derangements in beta cell function or resistance to the action of insulin was the principal element underlying the pathogenesis. Insulin resistance being the prime defect has been tracked back to the classic studies of Kerr and Himsworth (19). A combination of genetic as well as environmental factors contributed to the development of insulin resistance.

Insulin resistance:

Insulin resistance may be defined as an incompetence of insulin to yield its standard biological effects at usual circulating concentrations, which is habitually effective in normal subjects. There is loss of suppression of hepatic glucose production and also stimulation of glucose uptake by peripheral tissues is lost 20.Suppression of lipolysis in adipocytes by insulin is lost resulting in increased non-esterified fatty acids (NEFA) levels in plasma .This in turn results in stimulation of gluconeogenesis and triglyceride (TGL) synthesis in the liver. Inhibition of hepatic glucose production in the face ofhyperglycemia is absent leading to inappropriately high levels of glucose output from the liver21.

Obesity:

Obesity is strongly associated with type 2 DM. Individuals with BMI >35 kg/m^2 have forty times greater risk of developing the disease than individuals with BMI <25 kg/m^2. Truncal obesity, inherited or a result of sedentary lifestyle is associated with insulin resistance, the key factor in the pathogenesis of the disease21. Type 2 DM is known to occur as a late phenomenon in obese subjects and is heralded by normal glucose tolerance or IGT for many years. This is described by the overworked beta cell hypothesis, according to which during initial years of obesity the patients are normoglycemic due to high rate of insulin secretion by the beta cells22. Later on, the beta cells are not able to

compensate for the increased plasma glucose levels thereby resulting in the progression to type 2 DM from IGT.

Metabolic derangements in Type 2 DM:

Type 2 DM which is known for its complex polygenic nature is associated with dysregulation of carbohydrate, lipid and protein metabolism**.** Exposure to prolonged hyperglycemia causes irreversible changes to tissue metabolism.

Carbohydrate metabolism:

People with type 2 DM have high FPG levels and have a rapid change in glycemic levels following the ingestion of carbohydrate. This is possibly due to the decreased and delayed insulin secretion following ingestion of food23.Chronic hyperglycemia alone or in combination with elevated free fatty acids (FFA) impairs insulin secretion. Glucose is also an important regulator of its own metabolism. In the presence of basal insulin concentrations, an increase in plasma glucose stimulates glucose uptake and suppresses glucose production. The ability of glucose to regulate its own metabolism is impaired in type 2 DM. This is commonly referred to as a defect in "glucose effectiveness24."

Following glucose ingestion, there is reciprocal change in the concentration of insulin and glucagon. Marked hyperglycemia results from failure of glucagon secretion when secretion of insulin is decreased and delayed in type 2 DM. For non- diabetic individuals, due to the low insulin secretion in fasting state its role in suppression of gluconeogenesis

and glycogenolysis is negligible. Thus, the source of glucose in healthy individuals during fasting is from glucagon stimulated glycogenolysis.

During the fasting state in type 2 DM, signaling from glucagon activates glycogenolysis and gluconeogenesis resulting in the production of plasma glucose. Insulin supplemented exogenously, brings about the utilization of peripheral glucose. But there is insulin deficiency in the portal circulation, which ensues in lack of appropriate regulation of gluconeogenesis and glycogenolysis in liver. Exogenous insulin for individuals with type 2 DM in the fed state, is futile in quashing glucagon secretion through the physiological paracrine route. This causes a rise in the hepatic glucose production. The end result is postprandial hyperglycemia because the disappearance of glucose from circulation is exceeded by its appearance25.

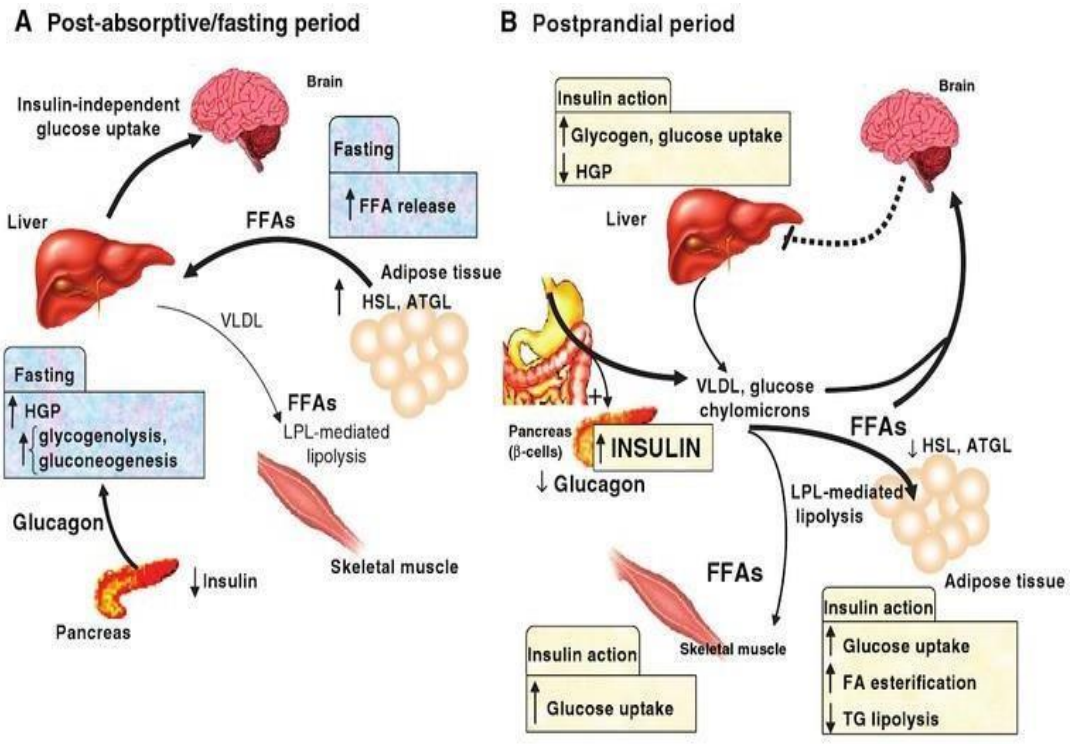


Figure 1: Glucose and Free Fatty Acid homeostasis

Source: Mark N. Feinglos, Mary Angelyn Bethel: Type 2 Diabetes Mellitus: An Evidence-Based Approach to Practical Management.

Protein metabolism:

Insulin is a vital hormone involved in the regulation of protein synthesis and breakdown. Deficit of insulin causes an increase in the levels of amino acids in the circulation due to escalated breakdown of protein along with decrease in the synthesis26. Signaling of insulin through the PI3K pathway is diminished in type 2 DM resulting in the block of insulin transport to destined tissues. Through the Akt pathway, PI3K activates mTORC1 and thereby plays a part in conveyance of amino acids to the muscles. But in type 2 DM due to the flaw in this pathway, synthesis of proteins in muscle cell is affected 27. The knowledge about the changes caused by type 2 DM on protein metabolism is vague when compared to that of type 1 DM. Catabolism of protein is limited by the residual secretion of insulin in type 2 DM. This helps in the conservation of the lean body mass. Nevertheless, in individuals with poor control of type 2 DM, there is a rise in the whole body nitrogen flux along with synthesis and breakdown of proteins26.

Lipid metabolism:

The foremost regulator of the release of FFA from the adipose tissue is hormone - sensitive lipase (HSL) and this is intricately sensitive to insulin. Insulin is the main hormonal regulator of lipolysis. The rise in the concentration of plasma glucose after a meal usually results in enhanced secretion of insulin, which thereby prevents lipolysis28.In individuals with type 2 DM, there is a surge in FFA concentration during the post-prandial and post-absorptive period. It is probable that the insensitivity of HSL to insulin in type 2 DM causes increased lipolysis. This results in increased levels of FFA which thereby kindles gluconeogenesis and TGL synthesis in the liver. Prolonged increase in FFA levels inhibit the secretion of insulin contradictory to its acute increase. Therefore, increased levels of FFA have been concerned with several of the metabolic abnormalities associated with type 2 DM29.

Lipoprotein abnormalities in DM:

DM is one of the most common source of derangement in lipid metabolism. Hypertriglyceridemia which is considered to be the paramount abnormality of lipid metabolism in type 2 DM is because of the increase of TGL-carrying lipoproteins namely chylomicrons and very-low-density lipoprotein cholesterol (VLDL-c). Amplified FFA flux into the liver with added decrease in the activity of lipoprotein lipase (LPL) is responsible for increased TGL synthesis. The abnormalities observed are surge in LDL-cholesterol (LDL-c) levels and decline in HDL-c concentration30.

Insulin resistance considered as the fundamental metabolic defect in type 2 DM causes release of FFA from the adipocytes. The FFA inturn enters the liver and kindles the assembly and secretion of VLDL-c which leads to hypertriglyceridemia. This VLDL-c stimulates the exchange of its triglycerides for cholesteryl esters from both HDL-c and LDL-c mediated by Cholesterol Ester Transfer Protein (CETP). Free apoA-I dissociated from HDL-c is promptly cleared from the circulation by excretion through the kidney. These changes decreases the availability of HDL-c for reverse cholesterol transport. Triglyceride enhanced LDL-c undergoes lipolysis and becomes smaller and denser. Both the decline in levels of HDL-c and the presence of small dense LDL-c are individual risk factors for cardiovascular disease31.

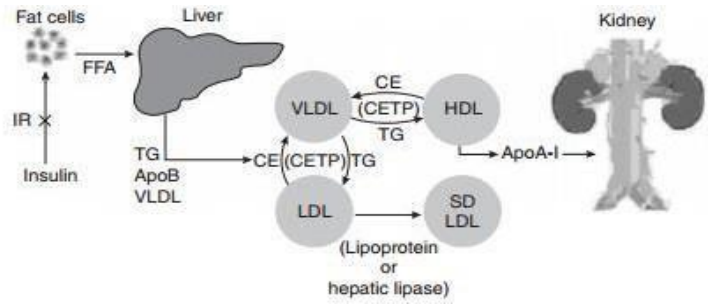


Figure 2: Lipid Metabolism in hepatic tissue (Source: Krentz AJ: Lipoprotein abnormalities and their consequences for patients with type 2 diabetes. Diabetes Obes Metab. 2003 ;5 (1):S19-27).

The distinctive feature of dyslipidemia in type 2 DM namely fasting and post- prandial hypertriglyceridemia results from above said increased synthesis of VLDL-c in the liver along with decline in LPL activity31. The capacity of triglyceride-rich remnant lipoproteins is more than LDL-c when concerned with the delivery of cholesterol to macrophages. Lipolysis results in the removal of the core of LDL-c and the resultant surface materials are transferred to HDL-c. This paves the way for LDL-c to be derived from VLDL-c. An important physiological role of LDL-c is delivery of cholesterol for the repair of cellular membrane and also to specified endocrine organs, for vitamin D and steroid hormones synthesis. Small and dense LDL-c particles are found to have more atherogenicity than their larger equivalents because of their liability to oxidation and their readiness to adhere and invade the arterial wall31.

HDL-c and reverse cholesterol transport:

Reverse cholesterol transport is considered to be the movement of cholesterol to the hepatocytes from various tissues and organs. The fate of cholesterol on entering the liver cell maybe its catabolism to bile acids, its excretion as free cholesterol into the bile, secreted as a component of lipoproteins into the plasma or esterification and storage in the hepatocytes. A portion of the bile acid and cholesterol eliminated into bile is lost through feces and is considered as the foremost loss of cholesterol and its metabolites from the body32.Cholesterol efflux from the cells is mainly facilitated by HDL-c which acts in juxtaposition with lecithin: cholesterol acyl transferase (LCAT)33.

The important steps involved in reverse cholesterol transport pathway is as follows: 1) HDL-c mediated uptake of cellular cholesterol 2) esterification of cholesterol present in HDL-c mediated by LCAT and 3) confiscation of HDL-c esters by the liver which will later on be metabolized for excretion into the bile34.

The nascent HDL-c considered as discoidal, pre-β-migrating complexes of phospholipid and apoA-I accepts cholesterol. These nascent HDL-c particles are synthesized by liver and small intestine apart from the formation during the metabolism of TGL-rich lipoproteins from excess surface material (phospholipid and soluble apoproteins). This in turn triggers cholesterol efflux in the macrophages and from the fibroblasts of sub-endothelial space. Externalized cholesterol is absorbed by nascent HDL-c and further undergoes esterification by LCAT to cholesterol esters. These cholesterol esters move into the core of the HDL-c particles due to their hydrophobic nature and the resultant particle is HDL335.

The class B scavenger receptor B1 (SR-B1) which has a dual role in metabolism of HDL-c is acknowledged as a HDL-c receptor .The major role of SR- B1in the tissues is, the acceptance of the effluxed cholesterol from the cells due to the action of HDL-c. This cholesterol is further transported to the hepatocytes for excretion through bile. In the liver and in steroidogenic tissues, SR-B1binds HDL-c via apoA-I, and cholesteryl ester is selectively delivered to the cells. HDL3, which is synthesized from discoidal HDL-c due to the feat of LCAT, accepts cholesterol from the tissues through SR-B1 and the cholesterol undergoes esterification by LCAT. This contributes to the formation of less dense HDL2 due to its increase in size. HDL3 is then renewed, after selective delivery of cholesteryl ester to the liver mediated by SR- B1 or by hydrolytic action of

hepatic lipase and endothelial lipase on HDL2 phospholipid and triacylglycerol. This interconversion of HDL2 and HDL3 is known as the HDL cycle. The free apo A-I released by these routes forms pre-β HDL after coupling with a least amount of phospholipid and cholesterol. Excess apo A-I undergoes destruction in the kidney36.

A second significant mechanism for reverse cholesterol transport involves proteins, namely the ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1). These

proteins are members of a family of transporter proteins that pair the hydrolysis of ATP to the binding of a substrate, thereby enabling its transport through the membrane. ABCG1 mediates transportation of cholesterol to HDL from the cells, whereas ABCA1 favours the efflux of cholesterol to particles poor in lipids like pre-β HDL or apo A-1, which are then converted to HDL3 by means of discoidal HDL36.

Cholesterol ester transfer protein (CETP) facilitates the equimolar exchange of cholesteryl esters from HDL for TGL in apoB100-containing lipoproteins namely VLDL-c and LDL-c. The delivery of the cholesteryl esters to the liver is mediated by low density-lipoprotein receptor (LDL-R). Then the cholesterol esters are transformed to bile salts to be eliminated via the gastrointestinal tract37.

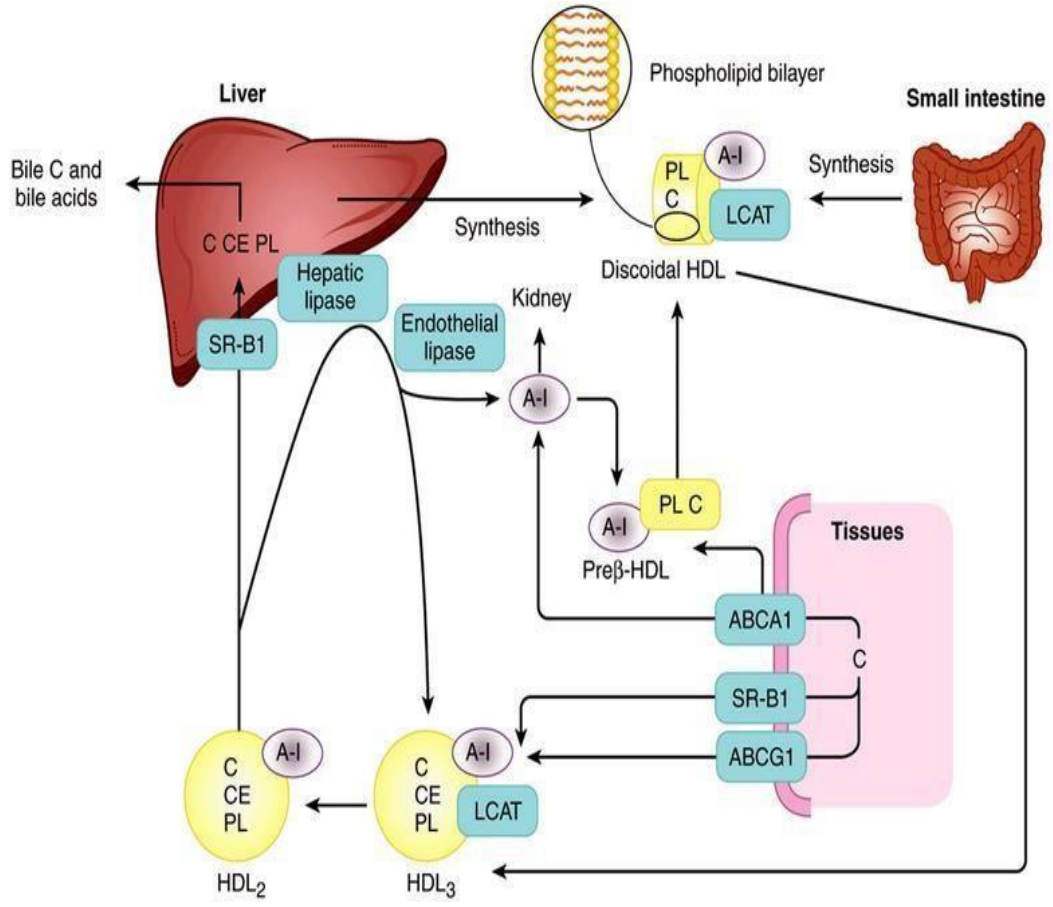


Figure 3: Metabolism of HDL in reverse cholesterol transport. Source: Harper's Illustrated Biochemistry, 29th Edition (2012).

ABCA1 gene:

Reverse cholesterol transport as aforementioned, is a pathway in HDL-c metabolism, which aids in the removal of excess cholesterol from the peripheral tissues to be transported to the hepatocytes to be dismissed from the body38.A protein known as ABCA1 is considered as the gatekeeper in reverse cholesterol transport39.

ATP Binding Cassette (ABC) transporters belong to a superfamily of proteins which utilize ATP as an energy source for transporting substrates across the cellular compartments40.The ABC transporters are categorized into 8 subfamilies from A to H, based on their amino acid sequences.

The ABCA1 gene in humans maps to chromosome 9q31 with its 50 exons spanning over 150 kbp41. ABCA1 is an integral protein with 2,261-amino acids. It has two transmembrane domains (TMD) each with six helices and also two nucleotide binding domains (NBD) in the cytoplasm. The nucleotide binding domains have 2 peptide motifs called Walker A and Walker B. An unique amino acid signature is present between the two Walker motifs, which is characteristic of ABC transporters family. In contrast to the other ABC transporters, in ABCA1 there are 2 regulatory domains with a hydrophobic segment located in the cytoplasmic region, one for each TMD and this favours the cooperative functioning of the two units of ABCA142.

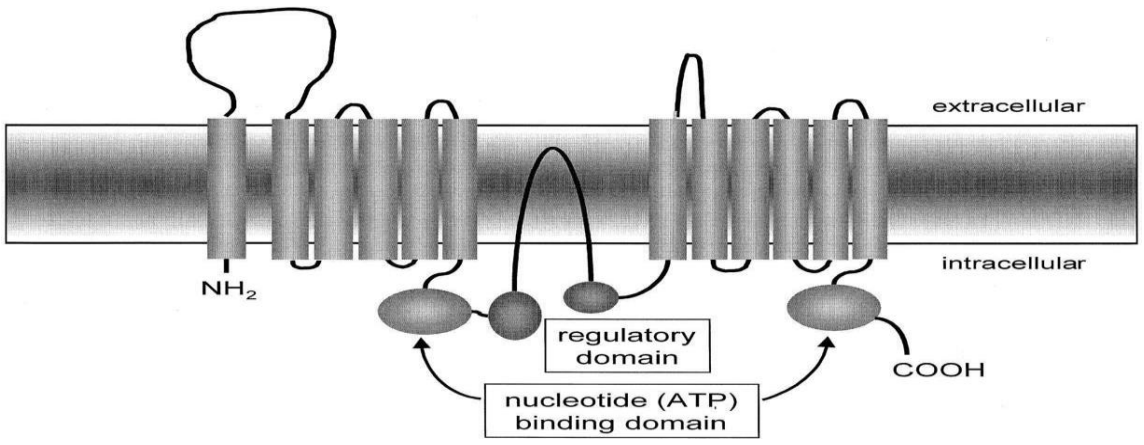


Figure 4: Transporter Protein ABCA1 Source: Fitzgerald M. L.,et al. ABCA1 contains an N-terminal signal-anchor sequence that translocates the proteins first hydrophilic domain to the exoplasmic space. J. Biol. Chem.2001; 276: 15137–15145.

ABCA1 has 2 extracellular domains, ECD1 and ECD2 which are liable for binding to apoA-I. ECD1 has 583 amino acids whereas ECD2 has 270 amino acids. The three dimensional structure reveals that ECD1 is placed above TMD2 and ECD2 is placed above TMD1. The extracellular domains form a flamed shaped structure which is stabilized by disulphide bonds. Regardless of this stability, the ECD has a hydrophobic hollow interior. This tunnel serves as a potential passage for the transportation of lipids to apoA-I from the interior of the membrane43.

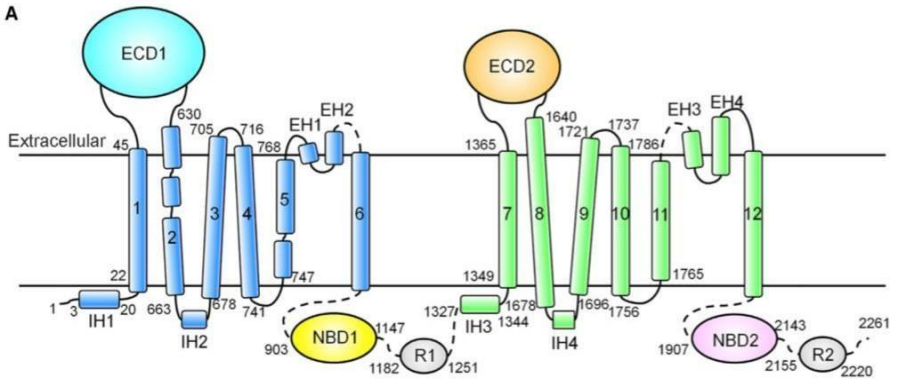


Figure 5: Amino Acids and peptides representation of ABCA 1 gene (Source: Qian et al. Structure of the Human Lipid Exporter ABCA1. Cell 2017;169: 1228– 1239).

The tunnel has both proximal and distal ends, with the opening at the distal end narrow than that of the proximal end .The proximal end opening is situated at a distance of 30Å from the transmembrane cavity. This structural arrangement shows that conformational change has to take place to facilitate the delivery of the lipids to the ECD from the transmembrane cavity43.

V-shaped α helical hairpins called extracellular helices, EH are present between trans membranes TM 5 & 6 of TMD1 and TM 11 & 12 of TMD2.These EH penetrate halfway

through the membrane. Apart from a narrow interface between TM5 and TM11 which is responsible for the contact of TMD1 and TMD2, all the other areas are exposed to the lipid bilayer. Thus this "outward facing conformation" favours the access of lipid substrates from inner side of membrane to the substrate binding site43.

The proposed mechanism is lateral access lipid- linked oligosaccharide (LLO) flopping by a protein called PgIK. The focal step in lipid flopping mechanism is the transport of the polar head across the hydrophobic barrier of membrane.A polar cluster present in TMD1 close to the inner membrane leaflet is responsible for binding the polar moiety of lipid. The conformational change caused in NBD due to ATP binding/hydrolysis and dissociation of ADP, leads to changes in TMD1 and TMD2 leading to delivery of

the polar head to a potential binding site in TMD located close to the outer leaflet. It is speculated that ABCA1 and lipid bilayer bind cooperatively with apoA-I and deliver the substrate for nascent HDL-c formation43.

Thus, ABCA1 plays an important role in the transportation of cholesterol and phospholipids from the cell, failure of which might lead to intracellular accumulation of lipids. This in turn leads to atherogenic changes, the most dreaded complication of type 2 DM.

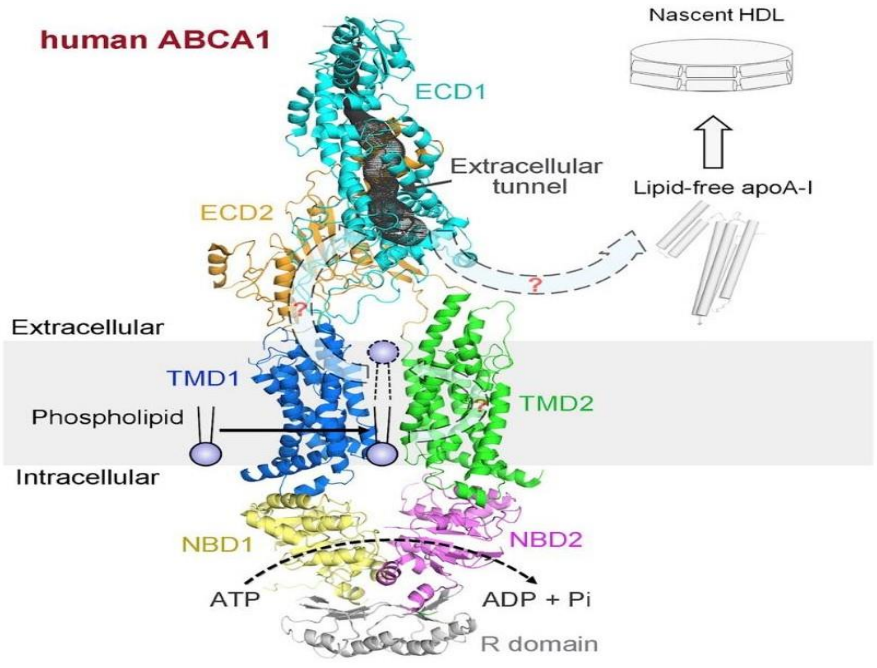


Figure 6: 3D model of ABCA1 protein (Source: Qian et al. Structure of the Human Lipid Exporter ABCA1. Cell 2017;169: 1228–1239).

In view of type 2 DM, there are few studies which were done to find out the association between ABCA1 gene polymorphism and the pathological mechanism underlying HDL-c dysfunction which is a significant derangement in lipid metabolism.

Polin et al44 have shown that there is a significant difference between cases and controls for R219K (rs2230806) polymorphism in Malayasian population.

Udita Singh et al45 have studied that ABCA1 gene(C69T) polymorphism has strong association with type 2 DM among central Indians.

Alharbi K K et al46 have conducted a study on the association of ABCA1 C69T gene polymorphism and the risk of type 2 DM in Saudi population.

MATERIALS AND METHODS

The study was conducted in department of biochemistry at Santosh Medical College, Ghaziabad. Ethical clearance for conducting the study was acquired from the Institutional Human Ethics Committee. An informed consent was acquired from the patients before collecting blood samples from them.

The study design is case control study in which type 2 DM patients (n =30) were selected as cases and control, respectively. The diagnosis of type 2 DM was based on the description in medical records using the American Diabetic association (ADA) criteria. Cases were selected from type 2 DM patients attending outpatient department of internal medicine Santosh Hospital, Ghaziabad. Patients satisfying the diagnostic criteria, inclusion criteria and exclusion criteria were explained about the study. After obtaining their consent, blood samples were collected, processed and stored for further analysis.

Individuals for control group (n=30) were selected mainly from the patients who came for master health checkup. Persons satisfying the criteria for control group were requested to participate in the study. Patient information sheet's were completed with the data comprising of height and weight etc. After getting the consent forms duly filled, samples were collected and stored for analysis after processing -20°C.

INCLUSION CRITERIA:

- Males and females
- Age 30 – 80 yrs
- Type 2 DM (ADA criteria)- maximum duration of 2 years on treatment (oral drugs /insulin)

EXCLUSION CRITERIA:

Subjects with:

- Malignancy and Acute illness
- Type 1 DM
- Type 2 DM with microvascular (Diabetic nephropathy, neuropathy, retinopathy) and macrovascular (Coronary artery disease, peripheral arterial disease,stroke) complications
- Genetic malformation
- Pregnancy
- Other endocrine abnormalities

Collection of blood samples:

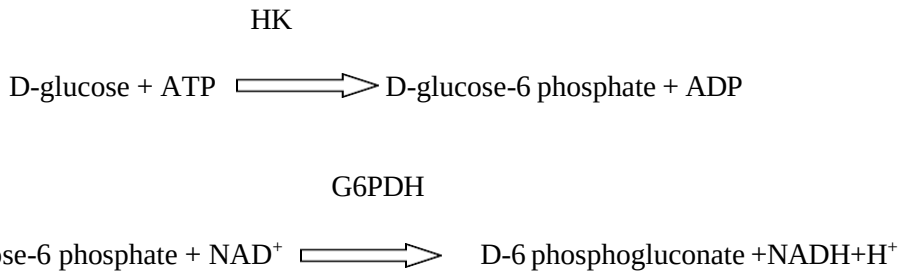
For both cases and control individuals, blood sample was collected in sodium fluoride vacutainer for fasting and post-prandial plasma glucose estimation and in EDTA vacutainer for DNA extraction. Whereas for lipid profile estimation, blood was collected in red topped serum tubes. The sample for DNA extraction was transferred to labelled sterile cryovial and stored at -80 degree celsius. The methods used for estimation of biochemical parameters are given below.

PLASMA GLUCOSE

Method: Enzymatic reference method with Hexokinase.

Principle: The phosphorylation of glucose by ATP to form glucose-6 phosphate and ADP is catalysed by Hexokinase (HK). This is followed by the action of another enzyme, glucose-6 phosphate dehydrogenase (G6PDH). This enzyme catalyzes oxidation of glucose-6 phosphate by NAD^+ to form NADH.

Reaction:



The NADH concentration is directly proportional to the concentration of glucose and the increase in absorbance is measured at 340nm.

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	340/409 nm
Test value	0-40 mmol/L
Unit	mmol/L

Conversion factor: mmol/L x 18.02 = mg/dL

GLYCOSYLATED HAEMOGLOBIN: (HbA1c)

Method: Turbidimetric inhibition immunoassay

Principle: EDTA tube collected whole blood specimen is hemolysed after collection. Total hemoglobin and HbA1c concentrations are determined. Total hemoglobin is measured colorimetrically. The percentage of HbA1c is determined immune-turbidimetrically. The ratio of both these levels gives the final percent HbA1c result.

EDTA collected blood is hemolysed with hemolysis reagent in the predilution cuvette. This leads to reduction in osmotic pressure which lyses the erythrocytes. Lysis of erythrocytes releases the haemoglobin. It is degraded by the proteolytic action of the enzyme pepsin. This

renders the beta-N terminal structures more available for the immunoassay. In the hemolysate, a colorimetric method helps in the determination of total haemoglobin. This is done on the basis of production of a brownish-green chromophore. This occurs in alkaline detergent solution using a cyanide free method. The intensity of color relates to the level of total hemoglobin in the sample. The concentration is calculated by sensing the raise in the absorbance at 552nm. A fixed factor that is obtained from the primary calibrator chlorohemin calculates the test results.

Turbidimetric method to measure HbA1c is done using monoclonal antibodies which are present attached to latex particles. The monoclonal antibodies bind the amino terminal fragments of HbA1c. The unbound free antibodies agglutinate with an artificial polymer. The change in turbidity is inversely related to the quantity of bound glycated proteins. This is measured turbidimetrically at 552nm. HbA1c result is given as percentage of HbA1c. It is evaluated from the ratio of HbA1c/Hb as given below:

According to IFCC

HbA1c (%) = (HbA1c / Hb) x 100 According to DCCT/NGSP

HbA1c(%) = (HbA1c/Hb) x 87.6 + 2.27

Test definition:

Mode of measurement	**Absorbance**
Mode of calculation of absorbance	Endpoint
Mode of reaction	D-R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	552 nm
Test value	1.4-4.7µmol/L (2.4-75.8mg/dL)
Unit	µmol/L

SERUM TOTAL CHOLESTEROL

Method: Enzymatic colorimetric test.

Principle: Cholesterol esters are cleaved by the action of cholesterol esterase (CE) to yield free cholesterol and fatty acids. Cholesterol oxidase (CHOD) in a second reaction helps in the formation of cholest-4-en-3-one and hydrogen peroxide by the oxidation of cholesterol. Peroxidase (POD) converts the hydrogen peroxide that is formed and helps in the oxidative coupling of phenol and 4-aminoantipyrine (AAP) forming a quinone-imine (red).

Reaction:

$$\text{Cholesterol esters} + H_2O \xrightarrow{CE} \text{Cholesterol} + \text{R-COOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CHOD} \text{Cholest-4-ens-3-one} + H_2$$

$$2H_2O_2 + 4\ \text{AAP} + \text{Phenol} \xrightarrow{POD} \text{Quinone-imine dye} + H_2O$$

The intensity of the red color produced is directly proportional to the serum cholesterol level and is determined by the increase in absorbance measured at 512 nm.

Test definition:

Mode of measurement	**Absorbance**
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	512/659 nm
Test value	0-20.7 mmol/L
Unit	mmol/L

Conversion factor: mmol/L x 38.66 = mg/dL

SERUM TRIGLYCERIDES:

Method: Enzymatic colorimetric method

Principle: Lipoprotein lipase (LPL) hydrolyzes the triglycerides to glycerol and fatty acids. Glycerol kinase (GK) catalyses the conversion of glycerol to glycerol-3- phosphate with the help of ATP phosphorylation. Glycerol phosphate oxidase (GPO) catalyses the oxidation of glycerol-3-phosphate to form dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase (POD) converts hydrogen peroxide, 4- chlorophenol and 4-aminophenazone to form a red-colored quinoneimine dye. The intensity of color is measured at 512 nm and the concentration of triglyceride is assessed in the sample.

Reaction:

$$\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol + Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3 phosphate + ADP}$$

$$\text{Glycerol -3 phosphate + O2} \xrightarrow{\text{GPO}} \text{Dihydroxy acetone Phosphate+H2O2}$$

$$\text{2H2O2 + 4 aminophenazone} \xrightarrow{\text{POD}} \text{Quinoneimine dye+4H2O+4 Chlorophenol}$$

Test definition:

Mode of measurement	**Absorbance**
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	512/659 nm
Test value	0-10 mmol/L (0-885 mg/dL)
Unit	mmol/L

Conversion factor: mmol/L x 38.66 = mg/Dl

SERUM HDL CHOLESTEROL:

Method: Homogenous enzymatic colorimetric assay.

Principle: With magnesium ions, dextran sulphate complexes with LDL, VLDL and chylomicrons which are water soluble and resistant to polyethylene glycol (PEG) modified enzymes. Cholesterol esterase and cholesterol oxidase associated with PEG to the amino groups is used to determine the cholesterol level using enzymatic methods. Cholesterol ester is partitioned into free cholesterol and fatty acids with the help of cholesterol esterase. Oxidation of cholesterol by cholesterol oxidase is done in the presence of oxygen to Δ^4-cholestenone and hydrogen peroxide. Hydrogen peroxide which is generated reacts with 4-aminoantipyrine and HSDA in the presence of peroxidase, forms a purple-blue dye. The cholesterol concentration is directly proportional to the intensity of blue color and it is measured photometrically.

Reaction:

$$\text{HDL Cholesterol ester} + H_2O \xrightarrow{\text{PCE}} \text{HDL Cholesterol} + RCOOH$$

$$\text{HDL-Cholesterol} + O_2 \xrightarrow{\text{PCO}} \Delta\text{ 4-cholestenone} + H_2O_2$$

$$2\,H_2O_2 + 4\text{ aminoantipyrine} + \text{HSDA} + H^+ \xrightarrow{\text{POD}} \text{Purple blue Pigment} + 4H_2O$$

HSDA = Sodium N (2 - hydroxy - 3- sulfopropyl) – 3,5-dimethoxyaniline

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	583/659 nm
Test value	0-3.12 mmol/L (0-120 mg/dL)
Unit	mmol/L

Conversion factor: mmol/L x 38.66 = mg/Dl

SERUM LDL CHOLESTEROL:

Method: Homogeneous enzymatic colorimetric assay.

Principle: Cholesterol esters and free cholesterol in LDL are measured on the basis of a cholesterol enzymatic method using cholesterol esterase and cholesterol oxidase in the presence of surfactants which selectively solubilize only LDL. The enzyme reactions to the lipoproteins other than LDL are inhibited by surfactants and a sugar compound. Cholesterol in HDL, VLDL and chylomicron is not determined.

Reaction:

$$\text{LDL-Cholesterol ester} + H_2O \xrightarrow{\text{Detergent cholesterol esterase}} \text{Cholesterol} + \text{Free fatty acid}$$

$$\text{LDL-Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \Delta^4\text{-cholestenone} + H_2O_2$$

$$\text{LDL-Cholesterol} + 2H_2O_2 + 4\text{ aminoantipyrine} + \text{EMSA}^a + H_2O + H^+ \xrightarrow{\text{POD}} \text{red purple pigment} + \text{N-ethyl-N-(3-methylphenyl)-N-succinylethylenediamine} + 5H_2O$$

Test definition:

Mode of measurement	**Absorbance**
Mode of calculation of absorbance	Endpoint
Mode of reaction	R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	583/659 nm
Test value	33/69
Unit	mmol/L

Conversion factor: mmol/L x 38.66 = mg/dL

ABCA1 GENE POLYMORPHISM:

The single nucleotide polymorphisms in ABCA1 gene namely R219K (rs2230806) and C69T (rs1800977) were selected for the present study based on population genetics. The mutation in R219K polymorphism was (G→A) whereas for C69T it was (C→T).

The following flowchart presents the overall steps involved in determination of ABCA1 gene polymorphism.

Collection of whole blood in EDTA containing vacutainer Isolation

↓

of DNA

↓

Genomic DNA quality check by 0.8% Agarose gel Electrophoresis

↓

External, F1, F2 and Reverse primers were designed for nucleotide sequence of interest

↓

Amplification of DNA sequence by PCR Separation of

↓

PCR product by 2% agarose gel electrophoresis

↓

Determination of the genotype

ISOLATION OF DNA:

The blood samples stored at -80 degree Celsius were thawed and genomic DNA was extracted using QIAamp DNA Blood Mini kit (Qiagen, USA). The following are the steps involved in the DNA extraction.

20 µL proteinase K was pipetted into the bottom of a 1.5 mL MCT

↓

200 µl of blood sample was added to the MCT

↓

Add 200 µL Buffer AL was added to the sample and it was mixed by vortexing for 15 sec

↓

MCT were incubated at 56°C for 10 min.

↓

MCT were briefly centrifuged to remove drops from the inside of the lid followed by addition of 200 µL of ethanol (96–100%) to the sample

↓

Samples were mixed again by pulse-vortexing for 15 sec, followed by briefly centrifuged the MCT to remove drops from the inside of the lid.

↓

Entire content was transferred onto QIAamp mini spin column in 2 ml collection tube Centrifuged at 8,000 rpm for 1 minute

↓

Flow through in the collection tube was discarded

↓

500µl of AW1 solution was added to the column and centrifuged at 8,000 rpm for 1 minute. Flow through in the collection tube was discarded

↓

500µl of AW2 solution was added to the column and centrifuged at 14,000 rpm for 1 minute. Flow through in the collection tube is discarded

↓

Column were placed back to a new 2 ml tube and centrifuged at 14,000 rpm for 1 minute to remove the any residual part from previous step

↓

Column were placed in a 1.5 ml eppendorf tube and 200µl of AE buffer is added to the column

↓

The 1.5 ml tubes were incubated at RT for 1 min and centrifuged at 8000 rpm for 1 min

↓

The eluted DNA quality was assessed by electrophoresis using 0.8% agarose gel.

↓

Genomic DNA was stored at -20^{0}C for further use

AGAROSE GEL ELECTROPHORESIS

Reagents required:

1. **TAE buffer 50 X stock:**
    - 24.2gmTris base
    - 5.7ml Glacial Acetic acid
    - 10ml 0.5M EDTA pH 8.0

- Dissolve in 100 ml Distilled water

2. TAE buffer 1X Tank buffer:

50X TAE buffer was diluted to 1X by adding distilled water. For eg: 10 ml 50X TAE + 490 ml distilled water makes 500ml of 1X TAE. This was used for preparation of agarose gel and for electrophoresis tank to run the gel.

3. 6X DNA loading dye

4. Ethidium bromide

5. Agarose powder

Gel preparation Protocol:

- 80 ml of 1X TAE buffer (based on gel template size) was taken in a conical flask and 0.64 g agarose (0.8% gel) was added, then the solution was heated well for the agarose to dissolve in it.
- Before that the gel template was sealed with cello tape and the well template was kept ready with comb to create wells.
- Then, add 0.2µl EtBr solution was added to the agarose solution, mixed well and poured into the sealed gel template. This is allowed to stand undisturbed for 15 minutes for solidification to occur.

Electrophoresis:

- 1X TAE buffer was poured in the tank (~600 ml) and the gel plate was kept inside the tank.
- Then, 1 µl of DNA loading dye, 1 µl of DNA (isolated) and 4 µl of nuclease free water were mixed well and loaded into the wells carefully.
- The voltage was set at 65V and electrophoresis was run it for 45 minutes.

- Gel Doc system was used to visually check the quality of DNA. The extracted genomic DNA was stored at -20 degree Celsius until further analysis.

POLYMERASE CHAIN REACTION:

The nucleotide sequence for the two SNPs of interest in ABCA1 gene was determined. Then forward and reverse primers for the flanking sequence and targeted primers from these single nucleotide sequences were designed using oligonucleotide calculator.

REQUIREMENTS:

SNPs	Sequences	Tm (^{0}C)	Product Sze
R219K (rs 2230806)			
External (E)	CTTATTCTTCCCATTCACCCTTTGG	56.0	**477 bps**
Wild (F1)	CTGCTGCAGCCAGTTTCTCC	55.9	**231 bps**
SNPs (F2)	CTGCTGCAGCCAGTTTCTCT	53.8	**231 bps**
Reverse (R)	TCAAAGCTGTATTGATATGAAGTTC TCC	55.5	
C69T(rs 1800977)			
External (E)	GCGTCTCTTTCTCCTACCCCTTGAC	61	**489 bps**
Wild (F1)	CGCTCGCAATTACGGGGTTTTGCCG	62.5	**201 bps**
SNPs (F2)	CGCTCGCAATTACGGGGTTTTGCCA	61	**201 bps**
Reverse (R)	CGGGCGGGGGAAGGGGAC	62	

- Taq DNA polymerase 2X master mix– Fermentas, USA (Composition – Tris-HCl pH 8.5, Ammonium sulphate, 3mM Magnesium chloride, 0.2% Tween, 0.4 mM of each dNTPS, 0.2units/µl Taq DNA polymerase)
- DNA sample
- Nuclease Free water

Amplification by PCR was carried out in DNA Thermal Cycler (T100 Biorad, USA). Reactions were performed in 20 µl as follows:

COMPONENTS	STOCK CONCENTRATION	FINAL CONCENTRATION	VOLUME (µl)
DNA template	500 ng/µl	500 ng	1
Buffer	10X	1X	2
dNTPs	10mM of each	200 µM of each	0.4
Taq polymerase	5U/uL	1 unit	0.33
Primer	20 pm	1 pm of each	1
$MgCl_2$	20mM	1.5mM	1.2
dH_2O	……	to make volume of 20 µL	14.07
		Total	20

The programme designed for the PCR reaction is as follows:

Steps	Temp (^{0}C)	Time	Cycle
Complete Denaturation	94	7 minutes	1
Denaturation	94	30 seconds	34
Annealing	58	45 seconds	34
Extension	72	30 seconds	34
Final extension	72	7 minutes	1

PCR amplification products were confirmed by 1.5% agarose gel electrophoresis. The PCR product size was determined using Gel Doc system by comparing with 100 bps DNA ladder which was run alongside the sample. For SNP R219K the product size was found to be 231 base pairs whereas for SNP C69T the product size was 201 base pairs. External primer as a positive control was also used for confirmation of the PCR.

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS:

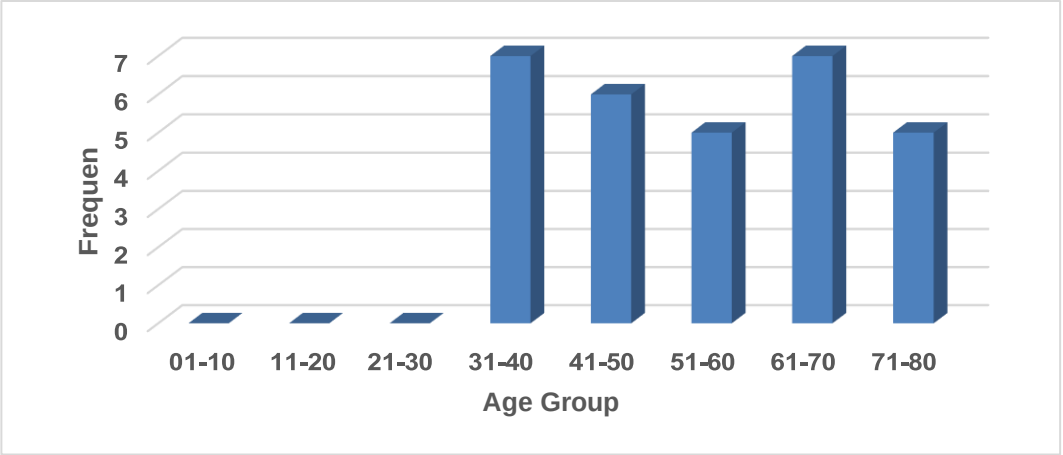
The data obtained after the computing BMI, WHR, estimation of FPG, post prandial glucose, lipid profile, HbA1c and genotype analysis were statistically analyzed using IBM SPSS software version 24.

The data distribution was displayed by bar diagram, histogram and pie chart. To find any statistically significant difference between the two groups in the distribution of the demographic characteristics and measured parameters, independent t-test was used. Additive model of odds ratio was used to determine the effect of dominant allele. The dependence of categorical variable was tested with Chi Square test. No significance was assessed at molecular level.

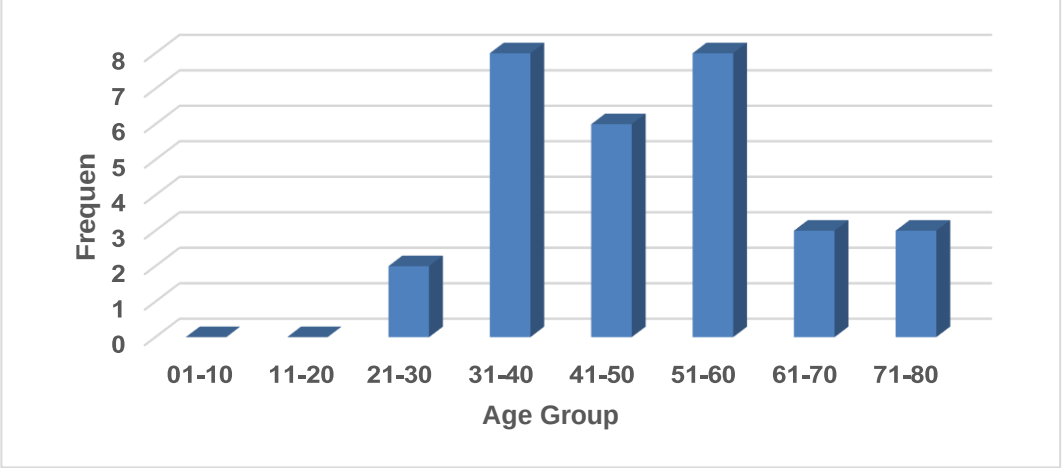
RESULTS

This research included 60 participants: 30 with type 2 diabetes and 30 without diabetes. The major goal of our study was to look into the relationship between anthropometric parameters, demographic variables, and risk factors in type 2 diabetes with ABCA1 polymorphism R219K (rs2230806) and SNP C69T (rs1800977) in the study patients.

Figures 1, 2, 3, and 4 show the distribution of the study population's demographic characteristics, including age, gender, and BMI.



(a)



(b)

Figure 1: Age Distribution among (a) Case and (b) Control Group

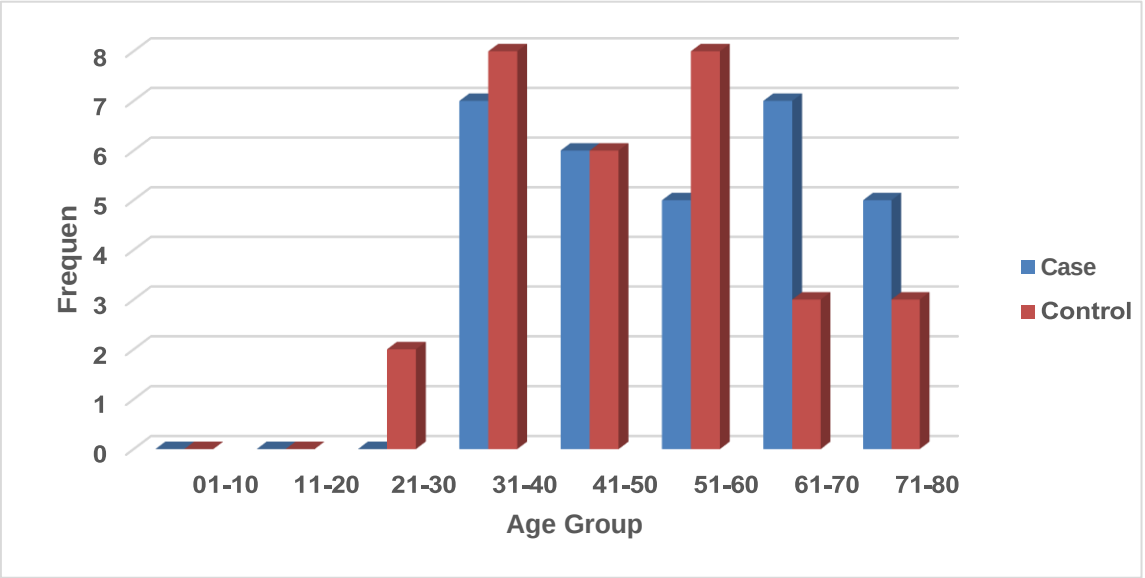


Figure 2: Age Distribution among study group

In terms of age and gender, both characteristics were found to be evenly distributed across cases and controls, meaning that the research groups were well matched for both parameters. This shows that the two groups are well-matched in terms of these criteria.

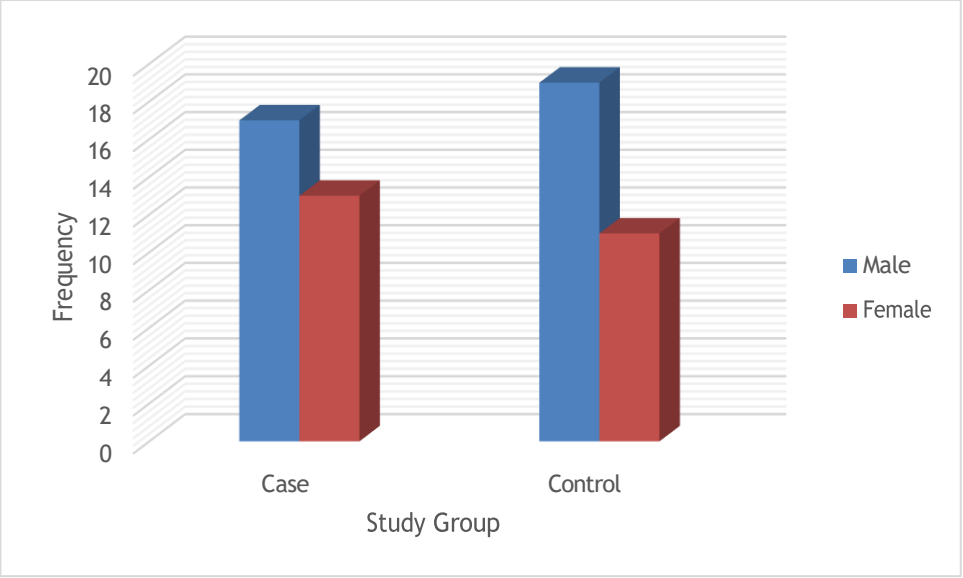


Figure 3: Gender based age distribution among study

TABLE 1: Clinical and biochemical characteristics among study groups

	Case N=30	Control N=30	p-value
Age	54.73±13.829	45.60±12.007	P<0.05
Height (cm)	166.77±7.3	169.67±8.503	P<0.05
Weight (kg)	78.53±8.127	77.67±11.133	P<0.05
BMI	28.220±2.1794	26.8753±2.47920	P<0.05
SBP	134.00±12.276	123.83±8.972	P<0.05
DBP	84.67±4.722	81.67±5.142	P<0.05
HbA1c	7.600±1.7156	5.317±0.2561	P<0.05
Total Cholesterol	138.60±45.128	158.97±38.071	P<0.05
TGL	151.747±69.4583	143.037±65.8213	P<0.05
HDL	38.403±4.9037	40.087±4.9693	P<0.05
LDL	70.1407±40.61391	91.4290±36.14541	P<0.05
VLDL	30.0193±13.80022	28.7180±13.22549	P<0.05
FBS	151.367±65.1926	92.243±8.7971	P<0.05

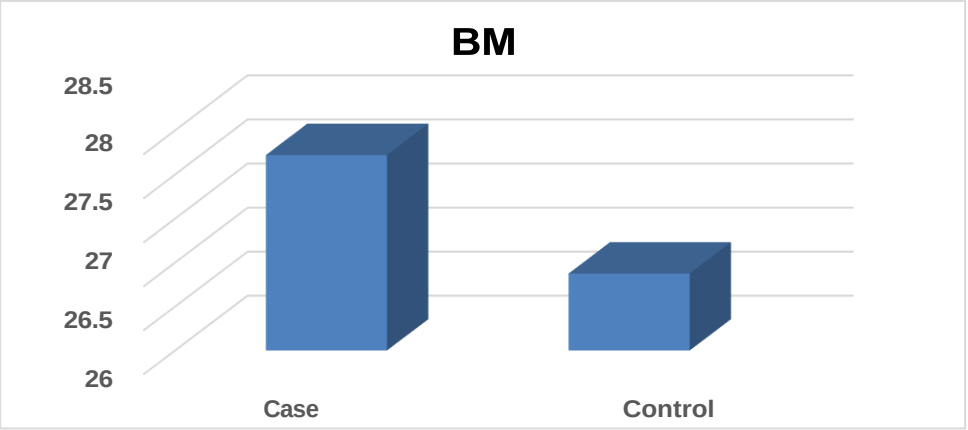


Figure 4a: Mean of BMI among study groups

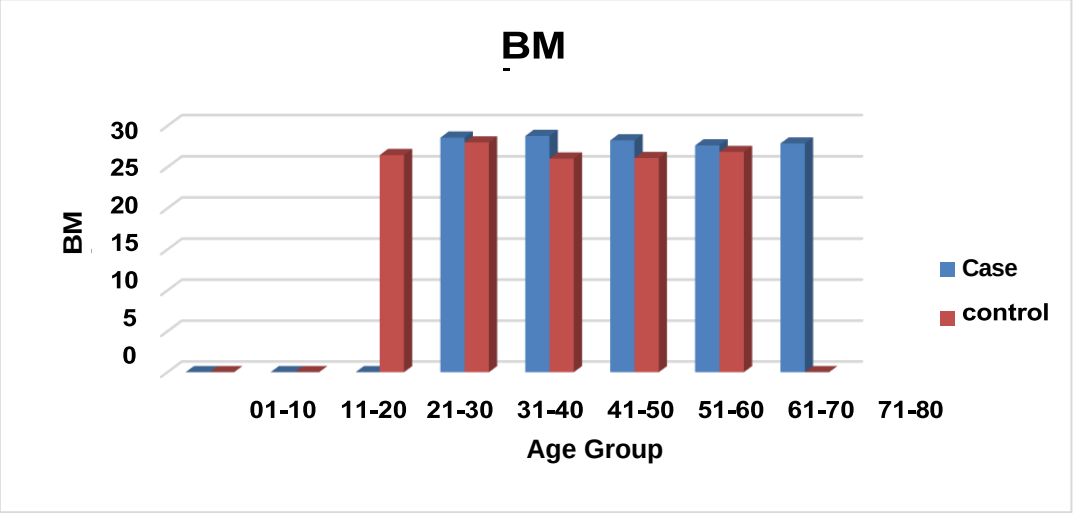


Figure 4b: BMI level among study groups

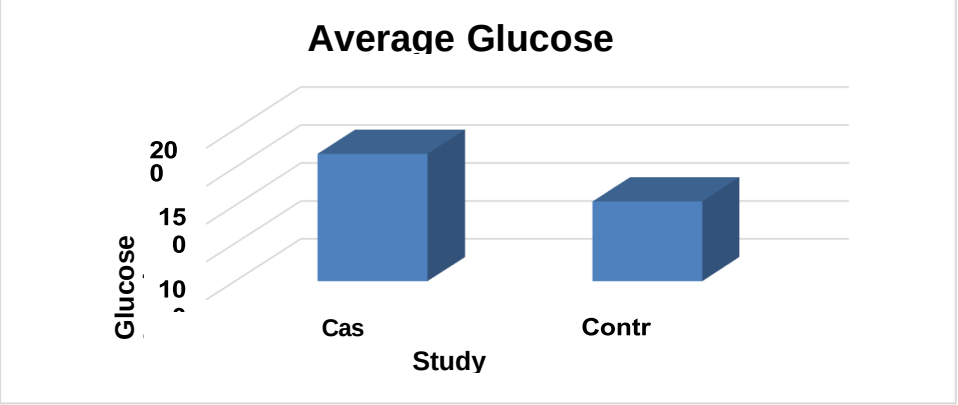


Figure 5a: Mean of Glucose level among study groups

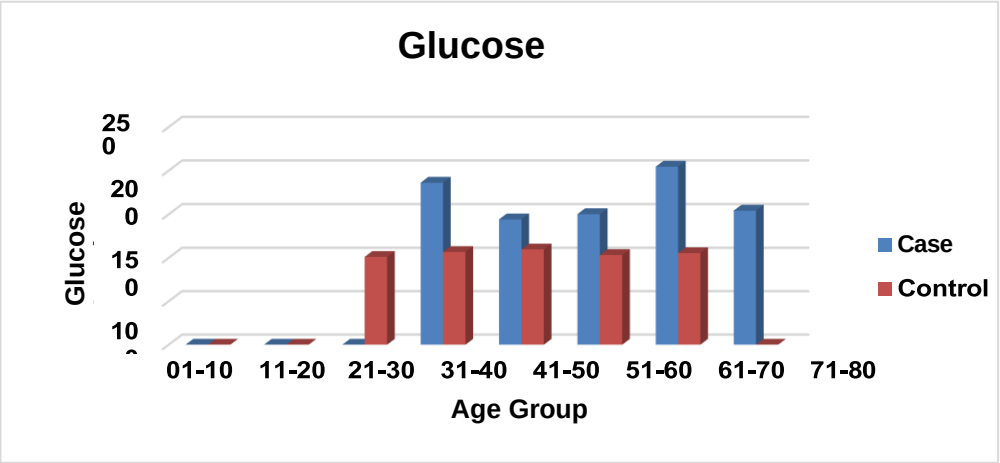


Figure 5b: Glucose level in different age among the study group

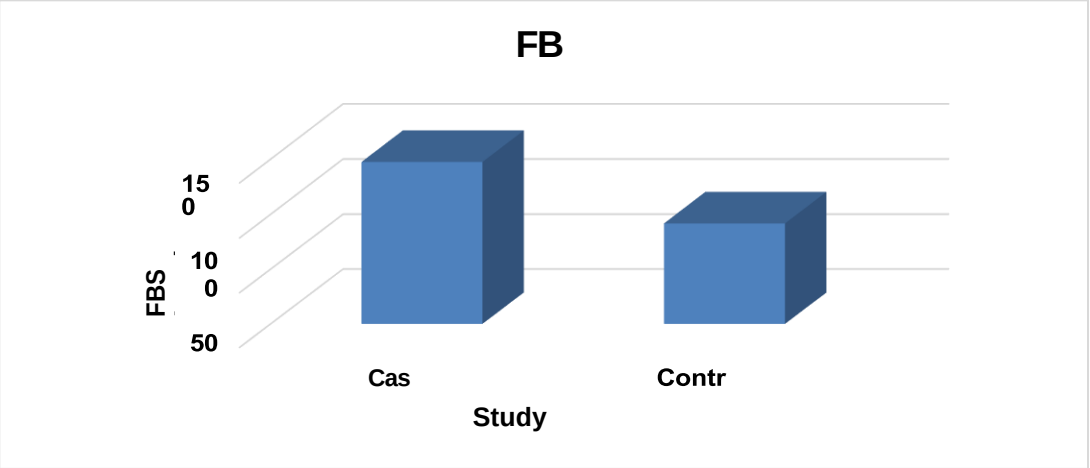


Figure 6a: Mean of FBS level among study groups

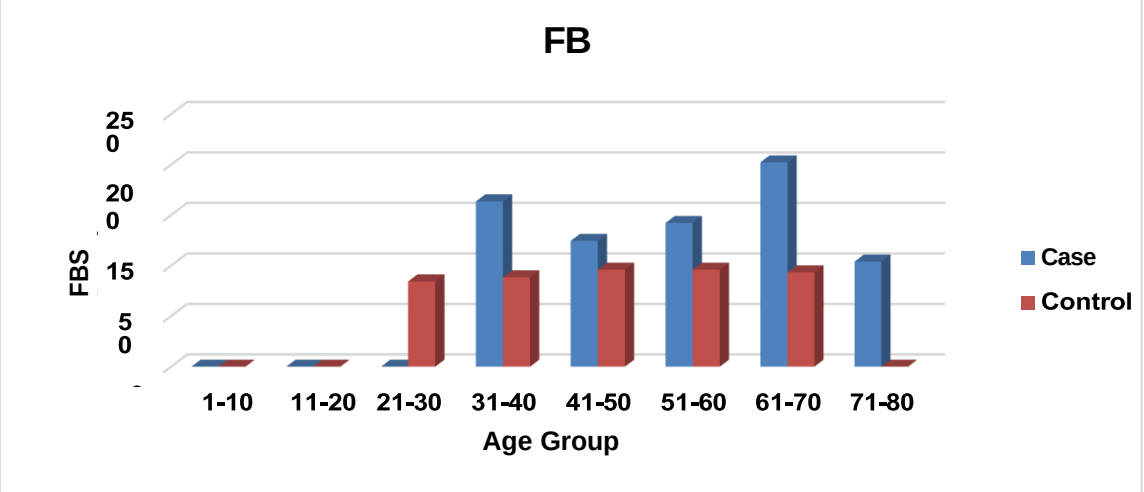


Figure 6b: FBS level in different age among the study group

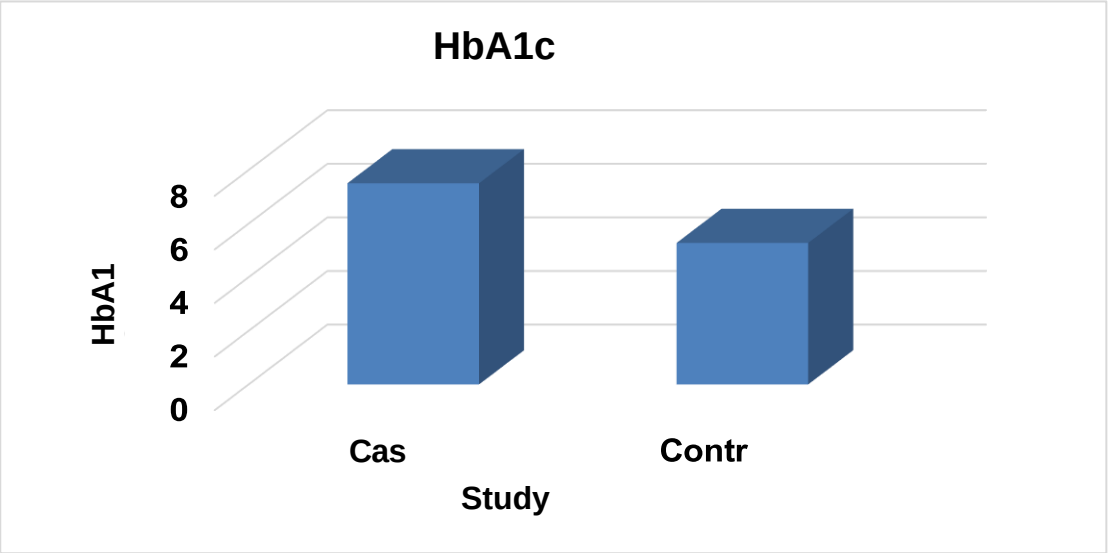


Figure 7a: Mean of HbA1c level among study groups

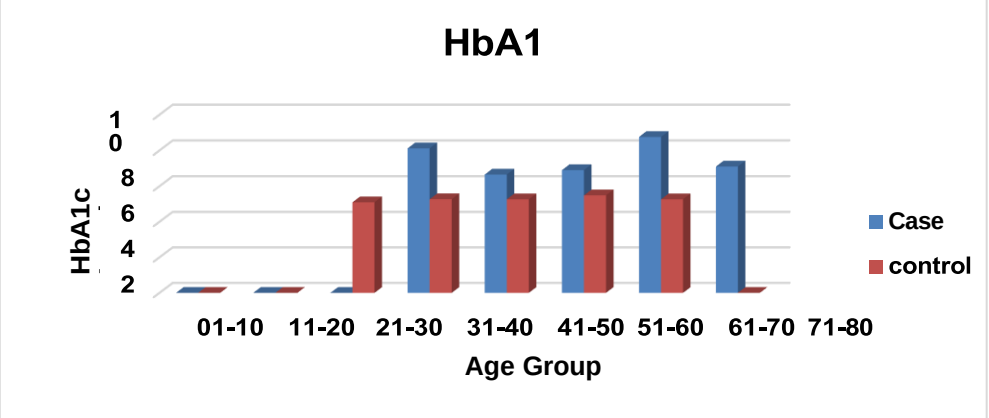


Figure 7b: HbA1c level in different age among the study group

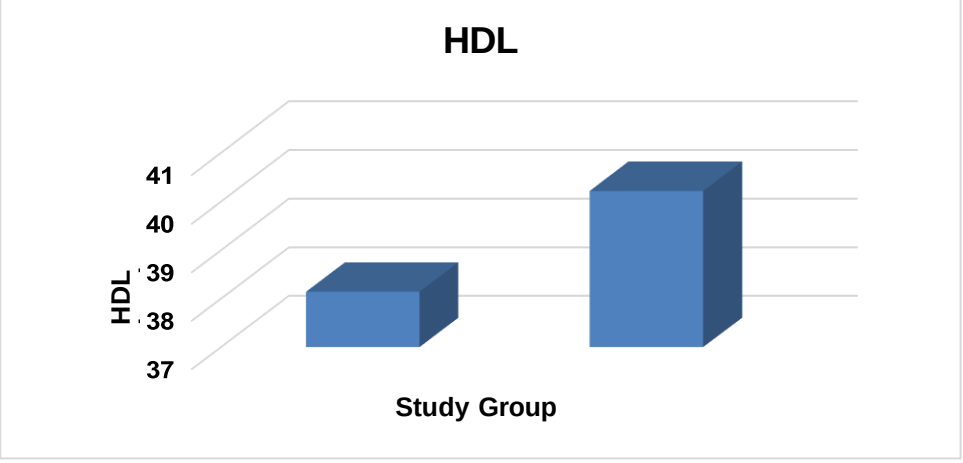


Figure 8a: Mean of HDL level among study groups

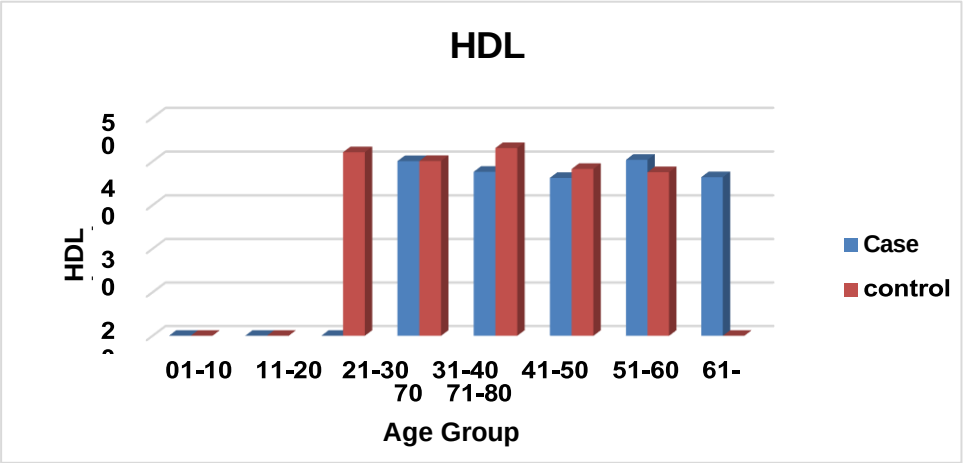


Figure 8b: HDL level in different age among the study group

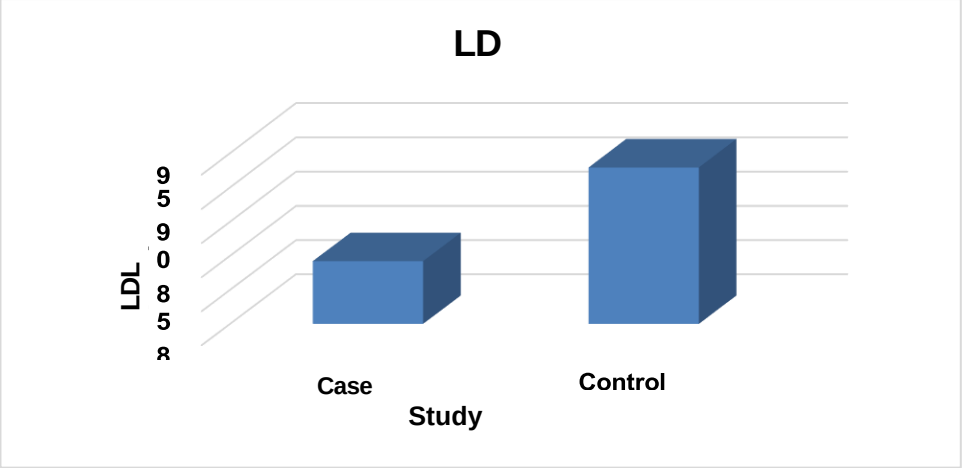


Figure 9a: Mean of LDL level among study groups

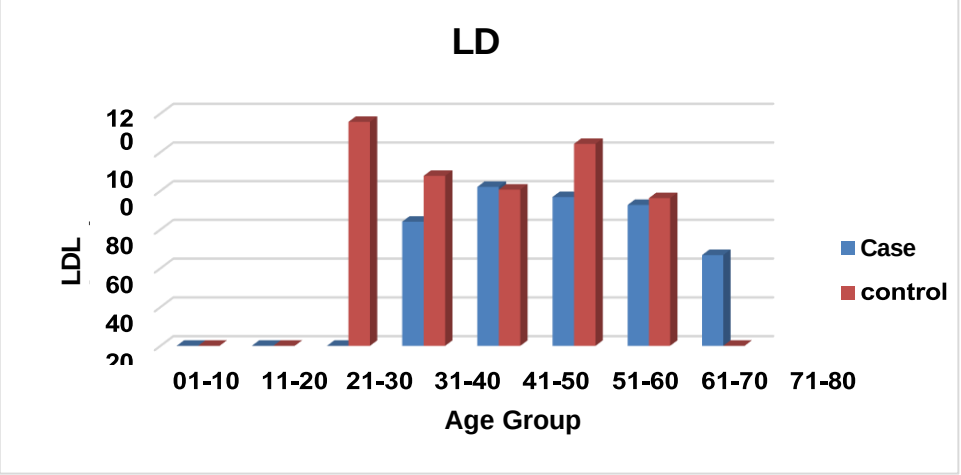


Figure 9b: HDL level in different age among the study group

The means of BMI in subject group was higher than control found. There was no significant difference in height and weight between case and control group. This suggests that the two groups are harmonized well with respect to these parameters.

Our investigation discovered that the mean FBS and HbA1c levels were higher in patients than in healthy controls. However, in terms of lipid profile, the control group had higher mean total cholesterol, LDL, and HDL-c levels than the patients (figures 5, 6, 7, and 8).

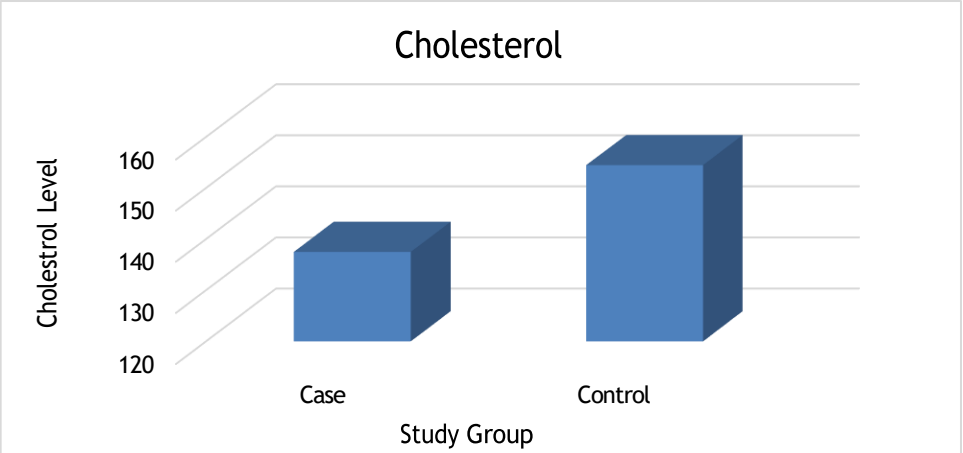


Figure 10a: Mean of Cholesterol level among study groups

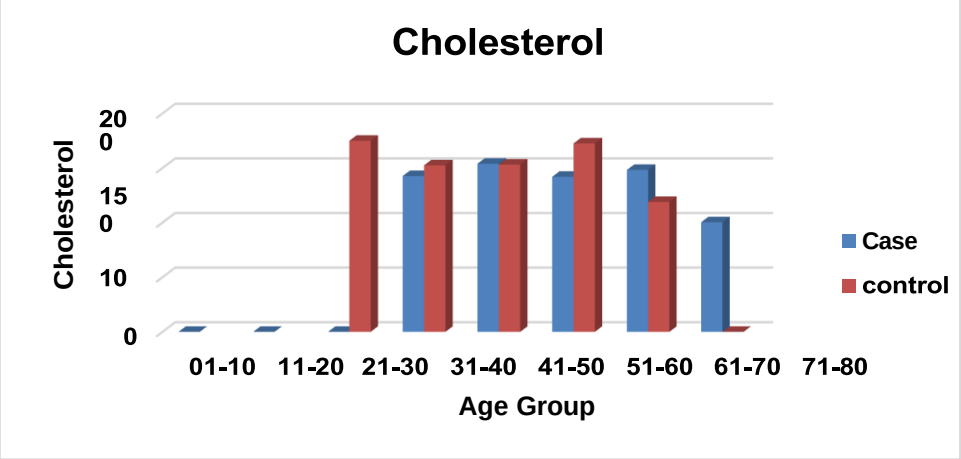


Figure 10b: Cholesterol level in different age among the study group

Only 19 (38%) of the 30 individuals with diabetes mellitus had a favourable family history. More than three-fourths of the patients, 88% (N=22), were on oral hypoglycemic agents. Furthermore, genotyping analysis of SNP R219K (rs2230806) and SNP C69T (rs1800977) of the ABCA1 gene revealed no statistically significant connection with type 2 DM patients. When numerous anthropometric and clinical data of patients were compared to both SNPs, there was no significant statistical difference except for age and HbA1c, which were statistically significant (P value 0.018 and 0.019, respectively) between the SNP R219K genotypes.

The frequency of Allele 'T' SNP for C69T (rs1800977) of ABCA1 gene is slightly higher (0.467) in case group when compared with control which shows 0.4 frequency of allele 'T' (figure 9).

Table: Genotype for C69T (rs1800977) among the case and control group

	Case	Control
CC	10	12
CT	12	12
TT	8	6

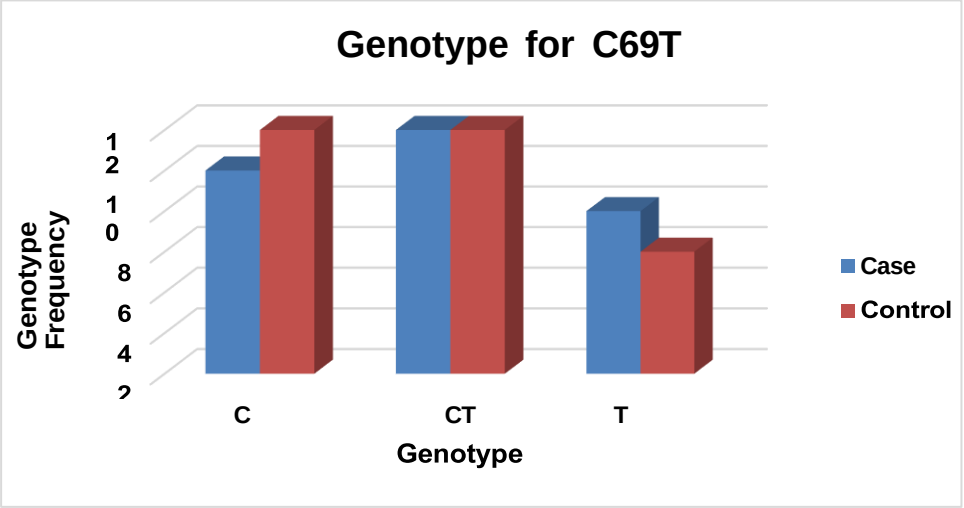


Figure 11a: Genotype C69T frequency among the study group

Table: Allele Frequency in case and control

	Case	Control
C	0.533333	0.6
T	0.466667	0.4

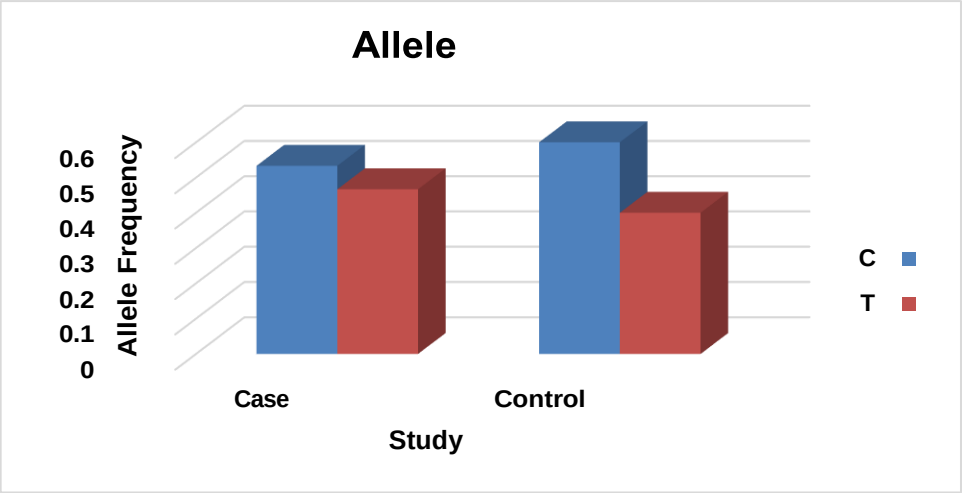


Figure 11b: Allelic frequency among the study group for SNP C69T (rs1800977)

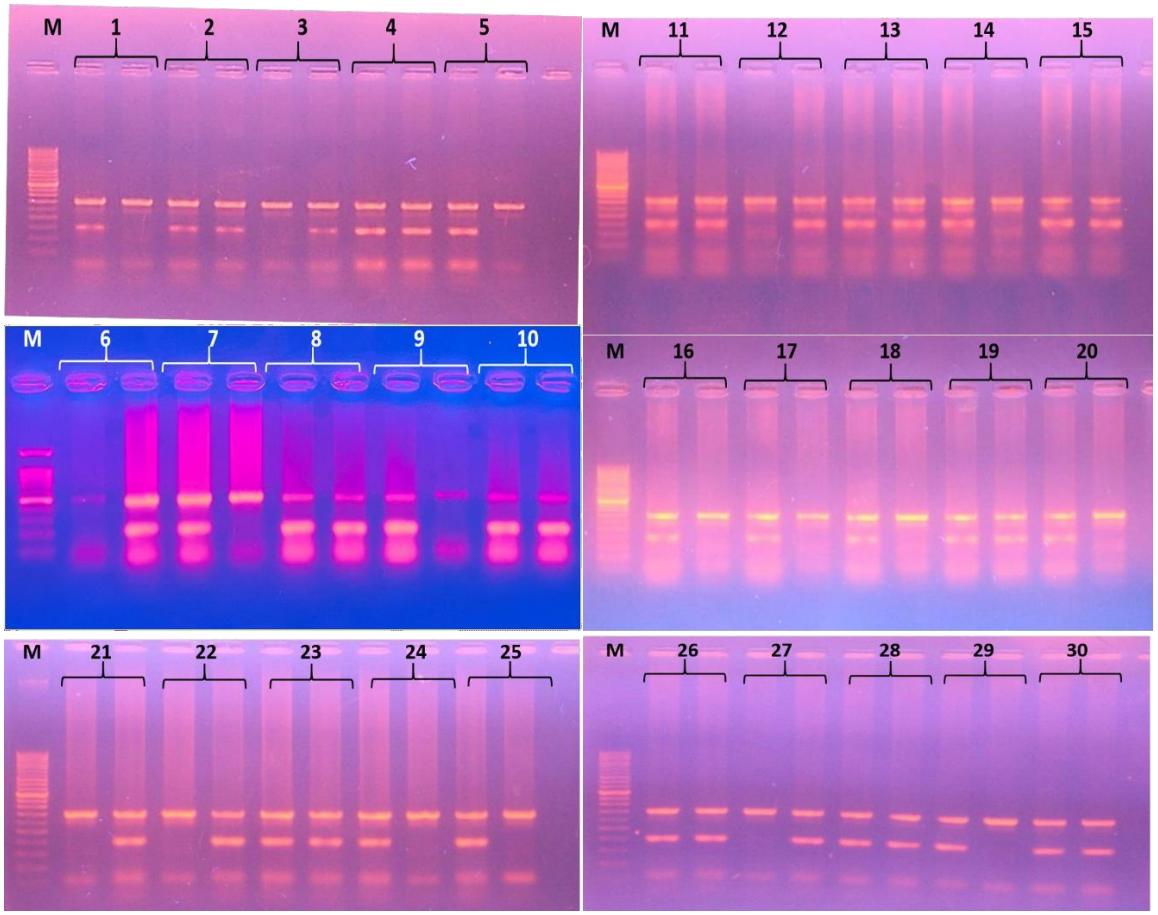


Figure 13a:Amplified PCR product SNP C69T (rs1800977)

Table: Genotype for G219A (rs2230806) among the case and control group

Figure 10a: Genotype G219A among the study group

	Case	Control
GG	9	13
GA	7	9
AA	14	8

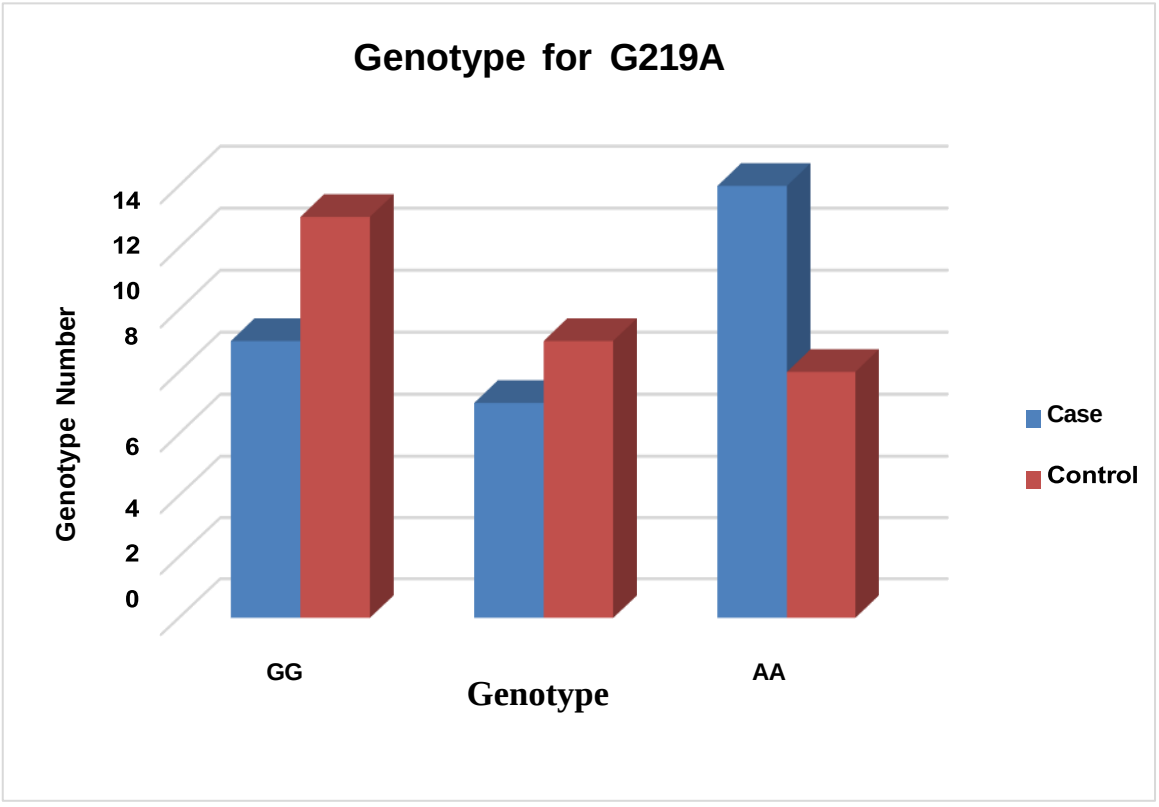


Figure 10a: Genotype G219A among the study group

Table: Allele G and A frequency in case and control

	Case	Control
G	0.416667	0.583333
A	0.583333	0.416667

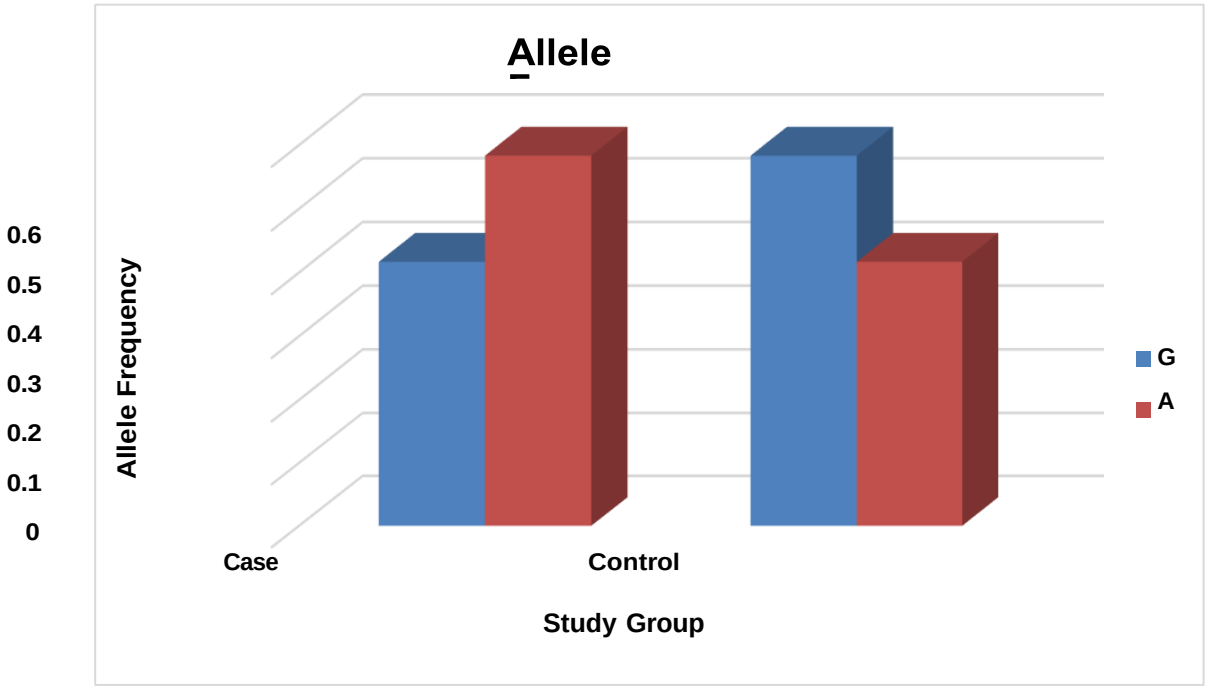


Figure 10b: Allelic frequency among the study group for SNP G219A (rs1800977)

The frequency of Allele 'A' SNP for G219A (rs2230806) of ABCA1 gene is significantly higher (0.583) in case group when compared with control which shows 0.417 frequency of allele 'A' (figure 10), which is related to disease.

DISCUSSION

The global prevalence of type 2 DM has increased exceptionally making it important to carry out research, to assess the risk factors and the multigenetic factors associated with type 2 DM. Type 2 DM is becoming a major public health problem mainly because of its complications[5]. One of the foremost complication due to type 2 DM is atherosclerosis, making it to be considered as cardiovascular risk equivalent.

According to the current evidence, it is believed that both environmental and genetic factors have almost equal contribution for the development of type 2 DM in an individual[46]. Several susceptibility genes linked with the metabolism of lipids are being investigated to assess their relationship with type 2 DM. One such gene is the ABCA1 gene, which is associated with HDL-c metabolism.

ABCA1 is a protein involved in reverse cholesterol transport of HDL-c metabolism. This pathway is recognized to protect against the development of atherosclerosis by clearing excess cholesterol from arterial cells[50].Polymorphism in ABCA1 gene causes HDL-c deficiency leading to accumulation of cholesterol in the tissue macrophages and the resultant atherosclerosis[51].

Our study was done to find the association of ABCA1 gene polymorphism with type 2 DM. Newly diagnosed type 2 DM patients(n=30) and non-diabetic healthy controls (n=30) were recruited for our study. The association of the genotype with demographic and biochemical characters of the cases were also studied.

Table 1 shows the age distribution among the study groups. The mean age±SD (years)

for the cases was 54.73±13.83 whereas for controls it was found to be 45.60±12.00. The difference between the mean age was not statistically significant (P>0.05) as shown in Table 1. This indicates that the two groups were matched well with respect to age. Sex distribution of the patients was found to be almost equal among the two groups (Figure 3).There was no statistical difference in BMI and WHR between cases and controls (P>0.05) as shown in Tables 2 & 3 respectively.

As per ADA criteria, individuals with HbA1c levels of 5.7%-6.4% fall into the prediabetes category whereas HbA1c>6.5% is diagnostic of type 2 DM. In the present study, the mean ±SD(%) values of HbA1c for cases and controls were 7.60±1.72 and 5.31±0.256 respectively. This clearly shows a noteworthy variation which was reinforced statistically (P<0.05) as shown in figure 7 and table 1. In addition to this the FPG levels among cases and controls had a significant difference with P<0.05(Table 4).

In the present study when the means of TC, TGL and LDL-c were assessed between the cases and controls, TC and TGL did not demonstrate any statistical difference. Nevertheless, LDL-c showed a significant difference(P<0.05) between the cases and controls as depicted in Table 1. In individuals with type 2 DM, clearance rate of LDL-c is reduced. This is because LDL-c binding to the receptor is under the stimulation by insulin. Hence due to the insulin resistance in type 2 DM there is a decrease in clearance of LDL-c thereby promoting dyslipidemia associated with type 2 DM[52].

According to NCEP ATP III guidelines, the optimal level for HDL-c is >50 mg/dL for both men & women. In our study, the mean±SD (mg/dL) of HDL-c among the cases

and controls were 38.403±4.9037 and 40.087±4.9693 respectively (Table1). This shows that HDL-c is below the optimal level in both cases and controls. Also, the mean±SD (mg/dL) of HDL-c was computed for males and females of cases and controls separately. The mean HDL-c levels of males and females in case group were 41.28 mg/dL and 38.62 mg/dL respectively. In control group, the mean was 42.6 mg/dL and 39.9 mg/dL among the males and females respectively. Hence there is a decrease in HDL-c the levels among the cases when compared to the controls. But this difference was not statistically significant (P>0.05) which might be due to the small sample size. When the levels of HDL-c decreases, it is considered as a risk factor for the development of cardiovascular complications of type 2 DM [44,45,49]. This decreased levels of HDL-c is promoted through the action of CETP which enhances the formation of TGL- rich HDL-c , thereby making it a very good substrate for hepatic lipase, an enzyme involved in HDL-c catabolism[53]. Due to this decrease in HDL-c levels and chronic inflammatory state of type 2 DM, HDL-c becomes pro-oxidant, pro-inflammatory and pro-thrombotic in nature. Thus, in spite of strict glycemic control and medications prescribed by the healthcare physicians, there is increased risk for the development of cardiovascular disease in type 2 DM which is evidenced by increased mortalities and morbidities.

Our study results shows that the means of liver function and renal function parameters, namely urea, creatinine, serum protein, serum bilirubin, SGPT, SGOT, ALP, GGT did not have statistical difference when compared among cases and controls.

Genotype Analysis:

In the current study, we chose two SNPs of ABCA1 gene namely R219K and C69T based on the population genetics and the clinical studies by Polin et al[44].,Udita Singh et al[45]., in which the presence of these two SNPs were found to be associated with type 2 DM among Malaysian and central Indian population respectively.

ABCA1 gene encodes a vital protein regulating cholesterol efflux from the cells to HDL-c. Many studies have reported that genetic variations in ABCA1 gene is associated with decreased serum HDL-c levels[47].This is because, it is speculated that ABCA1 and lipid bilayer bind cooperatively with apoA-I and deliver the intracellular cholesterol for nascent HDL-c formation[43]. Therefore a genetic variation in ABCA1 gene might disrupt the function of the protein resulting in decreased formation of HDL-c.

Our study results shows that there is a difference in the frequency of distribution of both C69T and R219K genotypes among the study groups. The genotypes of SNP R219K were AA (homozygous mutant), AG (heterozygous mutant) and GG (homozygous wild type) whereas the genotypes of SNP C69T were TT (homozygous mutant), CT (heterozygous mutant) and CC (homozygous wild type).

The frequency of R219K genotypes was AA (14 & 8), GG (9 &13) and AG (7 & 9) among cases and controls respectively. Similarly, the genotype distribution of C69T was TT (8 & 6), CC (10 & 12), CT (12 & 12) among cases and controls respectively. Thus our study results show that there is a noteworthy increase in the distribution of the homozygous mutant (AA & TT) among the cases, which was in accordance

with studies conducted by Polin et al[44] and Alharbi K K et al[46]., which demonstrated their association with decreased levels of HDL-c.

In our study, even though this difference in genotype frequency could not be substantiated statistically (P>0.05) the reason might be the small sample size (n=30 in each group) which was not large enough to demonstrate statistical difference. On increasing the sample size, there is a high likelihood of obtaining statistically significant results which will prove the association of ABCA1 gene polymorphism with type 2 DM.

Additionally, additive model for odds ratio was used to determine whether the presence of the dominant allele alone (A allele in R219K and T allele in C69T) is enough to cause pathological changes in HDL-c metabolism in type 2 DM. By this model, our study results showed that OR >1 implying that the presence of the dominant allele is a risk factor for the development of decreased levels of HDL-c in type 2 DM. This result is in concordance with the studies conducted by Polin et al[44] and Alharbi K K et al[46].

In our study, when the allele frequencies of C69T and R219K were computed, it revealed no significant difference amid the study groups (P >0.05). Further, the difference in sex distribution was assessed for C69T and R219K and it was not found to be statistically significant (P>0.05).

The association between the two SNPs and various parameters like age, BMI, WHR, FPG, HbA1c and lipid profile was assessed in the present study. There was no

substantial association (P>0.05) found for these parameters and the genotype distribution This was in accordance with the studies done by K K Alharbi et al[46] and Ergen et al[49].

The limitation with our study was the small sample size. Inspite of the presence of noteworthy difference in genotype distribution among cases and controls, the difference was not evident statistically due to the small sample size. The previous studies done to demonstrate the association of ABCA1 gene polymorphism in type 2 DM were done with a minimum sample size of 300. Hence, by increasing the sample size there is a high likelihood of obtaining statistically significant results.

CONCLUSION

1. SNP genotypes (C69T and R219K) of the ABCA1 gene differ between individuals with type 2 diabetes and healthy controls. However, due to the limited sample size, the difference in genotype frequency could not be statistically supported. Increased sample size increases the likelihood of achieving statistically significant results, providing solid evidence for the link of ABCA1 gene polymorphism in type 2 diabetes.
2. A dominant allele (A in R219K and T in C69T) is linked to lower HDL-c levels in type 2 diabetes. This demonstrates the connection of the ABCA1 gene polymorphism with type 2 diabetes.
3. There was no significant difference in allele frequencies of ABCA1 gene polymorphisms (R219K and C69T) between type 2 diabetes patients and healthy controls.
4. **Genetic Variants in ABCA1 Gene**

The genotyping results for the *ABCA1* gene revealed that the majority of participants carried the C allele for the C69T variant and the T allele for the R219K variant, with potential implications for cholesterol metabolism and cardiovascular health. Notably, individuals with the T allele in R219K may have altered lipid profiles, as observed in participants with higher LDL levels.

5. **Allele Frequency Calculation**:
    - Frequencies for each genotype were calculated.
    - C69T allele frequencies:
        - TT: 22/60 = 36.7%
        - CC: 14/60 = 23.3%
        - CT: 24/60 = 40%
    - R219K allele frequencies:
        - GG: 22/60 = 36.7%
        - GA: 16/60 = 26.7%
        - AA: 22/60 = 36.7%
6. **Statistical Tests**:
    - Chi-square tests were conducted to assess the significance of differences in genotype distribution.

Discussion

Analysis C69T single nucleotide polymorphism:

The genotype distribution shows that CT is the most prevalent genotype (40%), followed by TT (36.7%) and CC (23.3%).

The relative frequencies indicate a balanced distribution, suggesting that C69T may not be significantly associated with phenotypic variations in the studied population.

Analysis R219K single nucleotide polymorphism:

For R219K, the distribution of genotypes is relatively even, with GG and AA genotypes both at 36.7% and GA at 26.7%. The equal presence of GG and AA genotypes indicates potential significance, which may warrant further exploration regarding their functional implications.

Conclusion

The analytical validation of SNPs C69T and R219K demonstrates reliable genotyping results, with well-distributed genotype frequencies. No significant associations were observed based on the current data, but further investigation into their clinical relevance is recommended.

Recommendations

Further Studies: Expand the sample size and demographic diversity to validate findings.

Functional Studies: Investigate the biological implications of the identified SNPs.

Longitudinal Studies: Assess the impact of these SNPs over time in various populations

SUMMARY

Type 2 Diabetes Mellitus is a polygenic condition that affects glucose, lipid, and protein metabolism. Despite the fact that the number of research explaining the epidemiology of diabetes has increased dramatically over the last 20 years, diabetes mellitus remains a mystery due to its complex etiology.

ABCA1 is a protein subfamily that transports phospholipids and cholesterol from intracellular compartments to apolipoproteins with low lipid content. The ABCA1 gene is found on chromosome 9, region q31.1. ABCA1 gene variants have been linked to reduced blood HDL-c levels as well as changes in HDL-c quality.

This study was conducted to determine the connection of the ABCA-1 gene polymorphism with type 2 diabetes mellitus, which is accompanied by dysregulation in lipid metabolism. The study was designed as a case-control study, with 30 individuals per group. The genotype was determined using the ARMS-PCR technique, while biochemical markers such as FPG, HbA1c, and lipid profile were determined using an autoanalyzer.

The findings of our study revealed a significant difference in the genotype distribution of C69T and R219K among patients compared to controls. However, the difference was not statistically significant, which might be related to the limited sample size. Our findings revealed that the presence of a dominant allele (A allele in R219K and T allele in C69T) is a risk factor for the development of pathological alterations in HDL-c metabolism in type 2 diabetes. This suggests that there is a link between the ABCA1 gene polymorphism and type 2 diabetes, which may be confirmed by performing the study with a bigger sample size.

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PROFORMA

DEMOGRAPHIC DATA	
Name	**Mr./Mrs./Ms.:-**
Date of Birth:-	**Sex: Male/Female**
Weight (in Kg):-	**Height (in cm):-**
Address: **Street: _____ City: _____** **Stare: _____ Pin Code: _____**	
Mobile No.:	**Email:**
Education Status:	
Occupation:	
At which age T2DM diagnosed?	

SANTOSH DEEMED TO BE UNIVERSITY (GHAZIABAD)
INFORMED CONSENT

I……Son/Daughter/Wife of……. resident of……hereby declare that I give my full, free and voluntary informed consent in the thesis STUDY ON ASSOCIATION OF ATP-BINDING CASSETTE TRANSPORTER A1 (ABCA1) GENE POLYMORPHISMS IN TYPE 2 DIABETES MELLITUS AMONG NORTH INDIAN POPULATION. Investigator ……has informed me to my full satisfaction, in the language I understand, about the purpose, nature of study and various laboratory investigations for the study to be carried out. – I have been informed about the duration of the study and instructions to be followed during the study period.

I give full consent for being enrolled in the above study and I reserve my rights to withdraw from this study, whenever I wish without prejudice of my right to undergo further treatment at Santosh Hospital, Ghaziabad U.P

Patient's/ Patient's relative's signature Doctor's signature
Or thumb impression
Name:
Date:
We have witnessed that the patient signed the above form in the presence of his free will after fully understanding its content.

Signature of Witness
Name: Signature of Investigator

Name – Gaurav Maurya
Designation- Student
(Msc Medical Biochemistry, 2021-2024)
Contact No- 9889066116

संतोष डीम्ड यूनिवर्सिटी (गाज़ियाबाद)
सहमति पत्र (सुचित)

मैं.....बेटा/बेटी/

पत्नी……निवासी……..घोषण करता हूं कि शोधकता .....ने मुझे मेरी भाषा में अपने अध्ययन के उद्देश्य के बारे में और अध्ययन के दौरान जाने अपनायी जाने वाली विभित्र प्रयोगशाला जांच के बारे में सूचित कर दिया है। मुझे अध्ययनकी अवधि और उसके दौरान अपनाए जाने वाले निर्देश भी मेरी भाषा में समझा दिए गए हैं। मैं उपरोक्त अध्ययन में बिना किसी दबाव के दाखिला ले रहा हूँ और पूर्ण सहमति दे रहा हूं। सन्तोष अस्पताल, गाजियाबाद में अपने आगे के उपचार पर बिना प्रतिकूल प्रभाव डाले इस अध्ययन से और प्रयोगशाला जांच की प्रकृति से में जब चाहूँ बाहर जाने के अपने अधिकार को सुरक्षित रख रहा हूं।

रोगी के रिश्तेदार के हस्ताक्षर डॉक्टर के हस्ताक्षर।
नाम
दिनांक:

मेरी उपस्थिति में मरीज ने पूरी तरह से डाकटर के बताए गए अध्ययन और निर्देशों को समझने के बादअपनी स्वतंत्र इच्छा से ऊपर के फार्म पर हस्ताक्षर किए हैं।

हस्ताक्षर

नाम: .....
पदनामः .....
संपर्क नंबर: ……

DETAILS OF PATIENT INFORMATION SHEET
HUMAN RESEARCH PROPOSAL

1. Identifying Data

Name of Researcher: Gaurav Maurya
Department: Biochemistry
Title of Project: STUDY ON ASSOCIATION OF ATP-BINDING CASSETTE TRANSPORTER A1 (ABCA1) GENE POLYMORPHISMS IN TYPE 2 DIABETES MELLITUS AMONG NORTH INDIAN POPULATION

2. Description of Human Subjects Safeguards and Rights:

a) Possible psychological of physical risk of discomfort, involved in research If Yes/No (if no, skip time B)
If yes, answer the following
Why necessary?
   1. To have the common disease
   2. Specificity and sensitivity of the treatment

b) Make sure the following questions are answered in your research proposal on collection of Data:
   1) When will it take place?
   2) What length of time needed for complete examination of the patient?
   3) What period of the time needed?
   4) Which specific place within institution?
   5) How much population required including number of subjects?
   6) Name of instrument to be used for data collection?
   7) Methodology to be used (write details)?

c) Will subjects be given research results if they so request? Yes / No

LETTER OF ETHICAL CLEARANCE FOR DISSERTATION FROM REGISTRAR OFFICE

SANTOSH
Deemed to be University
(Established u/s 3 of the UGC Act, 1956)

F. No. SU/R/2024/1344[7] Dated: 17.05.2024

Subject: Grant of Ethical Clearance for **Dissertation Protocol** Topic **"Genetic plymorphism in ATP Binding cassette Protein Gene",** submitted by **GAURAV MAURYA,** student of M.Sc. Medical BIOCHEMISTRY, 2021 Batch.

*****

With reference to the request for grant of Ethical Clearance for the Dissertation Protocol entitled **"Genetic plymorphism in ATP Binding cassette Protein Gene", GAURAV MAURYA** is informed that the **Dissertation Protocol** submitted by him/her was considered by the **Screening Committee** of the **Allied Health Sciences** in its meetings held on 07.02.2024 and 29.02.2024. The recommendations of the **Screening Committee** were considered in detail by the **Institutional Ethics Committee** in its meeting held on **15.05.2024** and the same was **approved** by the **Ethics Committee** of the **Santosh Deemed to be University, Ghaziabad, Delhi NCR**.

He /She is informed accordingly for further necessary action.

17/5/24

[Dr. EZHILRAJAN. K]
REGISTRAR

SANTOSH DEEMED TO BE UNIVERSITY REGISTRAR GHAZIABAD, NCR DELHI

GAURAV MAURYA
M.Sc. Medical BIOCHEMISTRY.

Copy to:

1. The Vice Chancellor
2. The Dean – Academics
3. The Dean, SMC&H
4. The Dean – Research
5. The Director - IQAC
6. HOD/ Course In-charge of the concerned Department.

STATISTICIAN CERTIFICATE

This is to certify that the statistical analysis component of the thesis titled "STUDY ON ASSOCIATION OF ATP-BINDING CASSETTE TRANSPORTER A1 (ABCA1) GENE POLYMORPHISMS IN TYPE 2 DIABETES MELLITUS AMONG NORTH INDIAN POPULATION" done by Gaurav Maurya in the Department of Central Research Facility, Santosh Medical College and Hospital, Ghaziabad has been conducted under my direct guidance and supervision.

All the statistical calculations of this study are authentic and correct to the best of my knowledge.

Wish him all the success in his life.

Dr. Amod Kumar
Assistant Professor cum Statistician
Department Of Community Medicine
Santosh Deemed To be University, Ghaziabad.
Email : Amod.ism01@gmail.com

PLAGIARISM CERTIFICATE

STUDY ON ASSOCIATION OF ATP-BINDING CASSETTE TRANSPORTER A1 (ABCA1) GENE POLYMORPHISMS IN TYPE 2 DIABETES MELLITUS AMONG NORTH INDIAN POPULATION

ORIGINALITY REPORT

2%
SIMILARITY INDEX

PRIMARY SOURCES

1	Batoul Sadat Haerian, Monir Sadat Haerian, Azam Roohi, Hassan Mehrad-Majd. "ABCA1 genetic polymorphisms and type 2 diabetes mellitus and its complications", Meta Gene, 2017 Crossref	48 words — 2%

EXCLUDE QUOTES	ON	EXCLUDE SOURCES	< 10 WORDS
EXCLUDE BIBLIOGRAPHY	ON	EXCLUDE MATCHES	< 14 WORDS

MASTER CHART

Master chart for demographic parameters of T2DM patients

Case Sample ID	Gender	Age	Height (cm)	Weight (kg)	BMI	SBP	DBP
T2DM1	Male	63	170	88	30.4	120	80
T2DM2	Male	71	160	68	26.6	115	75
T2DM3	Male	50	155	72	30	130	80
T2DM4	Male	60	168	90	31.9	140	85
T2DM5	Male	32	158	70	28	125	80
T2DM6	Female	40	172	95	32.1	130	85
T2DM7	male	40	162	76	29	145	85
T2DM8	female	43	175	85	27.8	135	85
T2DM9	female	62	158	67	26.8	150	95
T2DM10	male	40	160	82	32	120	80
T2DM11	Female	73	165	72	26.4	140	85
T2DM12	Male	60	175	78	25.5	160	90
T2DM13	Male	65	158	70	28	165	95
T2DM14	Male	33	160	65	25.4	130	85
T2DM15	male	64	180	85	26.2	125	80
T2DM16	Male	77	170	90	31.1	120	80
T2DM17	male	51	172	75	25.4	145	90
T2DM18	male	48	170	80	27.7	130	85
T2DM19	female	40	160	70	27.3	120	80
T2DM20	female	80	162	78	29.7	130	85
T2DM21	female	62	168	85	30.1	140	85
T2DM22	female	53	170	82	28.4	150	90
T2DM23	female	65	160	66	25.8	145	90
T2DM24	male	75	175	78	25.5	125	80
T2DM25	male	43	172	80	27	135	85
T2DM26	female	50	170	90	31.1	120	80
T2DM27	female	59	155	72	30	130	85
T2DM28	Male	36	180	85	26.2	130	85
T2DM29	female	42	168	82	29.1	140	90
T2DM30	female	65	175	80	26.1	130	85

Master chart for biochemical parameters and R219K & C69T genotype of T2DM patients

Case mple ID	HbA1c	Total Cholestrol	TGL	HDL	LDL	VLDL	FBS	ABCA1 genotype C69T	ABCA1 genotype R219K
T2DM1	7.7	98	112.9	35	40.42	22.58	212.2	TT	GA
T2DM2	6.5	115	109	35	58.2	21.8	103.1	CC	AA
T2DM3	7.3	262	191.3	40	183.74	38.16	170.2	CT	AA
T2DM4	7.8	144	194.7	36	69.06	38.94	175.7	CC	GG
T2DM5	8	132	82.5	35	80.5	16.5	156.9	CC	GA
T2DM6	9.8	216	191.9	42	135.62	38.38	239.9	CC	GG
T2DM7	6.7	130	158.7	43	55.26	31.74	110.4	CT	GA
T2DM8	6.5	132	231.3	41.8	43.94	46.26	105.5	CC	AA
T2DM9	5.9	163	126.1	44.5	93.28	25.22	120.2	CT	AA
T2DM10	9.7	130	247	50.5	30.1	49.4	158.2	CT	AA
T2DM11	8.4	115	73.8	42	58.24	14.76	77.3	CT	AA
T2DM12	6.4	95	133	29.4	39	26.6	121.9	CC	AA
T2DM13	7.1	224	200	38	154	31	157	TT	GG
T2DM14	6.5	98	78.4	35	48.12	14.88	138.1	TT	GG
T2DM15	6.7	193	147.1	51.1	112.48	29.42	108.1	CT	GA
T2DM16	6.7	112	89.6	38	56.08	17.92	112.3	CT	AA
T2DM17	6.5	144	116.8	35	85.64	23.36	134.6	CC	GG
T2DM18	6.7	85	99.2	30	35.16	19.84	113	CC	AA
T2DM19	6.6	120	193	35	46.4	38.6	112	TT	AA
T2DM20	7.4	75	87.7	32	25.46	17.54	108.9	CT	GG
T2DM21	11.6	110	241.5	38	23.7	48.3	268.4	CT	GA
T2DM22	6.1	159	145.3	40.7	89.24	29.06	121.6	TT	AA
T2DM23	9	123	120.6	38	60.88	24.12	147	TT	AA
T2DM24	6.5	85	69.4	35	36.12	13.88	118.7	TT	GG
T2DM25	6.7	205	132.1	38	140.58	26.42	124.2	CC	GA
T2DM26	6.3	105	184.8	38	30.04	36.96	106.3	CT	AA
T2DM27	7.7	168	134.7	40.1	100.96	26.94	157.7	TT	GG
T2DM28	9.5	173	404.3	40	52.14	80.86	230.2	CT	GA
T2DM29	6.4	120	115.6	38	58.88	23.12	128.4	CC	AA
T2DM30	13.3	127	140.1	38	60.98	28.02	403	CT	GG

Master chart for demographic parameters of healthy controls

Control Sample ID	Gender	Age	Height (cm)	Weight (kg)	BMI	SBP	DBP
C01	Female	44	175	80	26.1	120	80
C02	Male	35	180	90	27.8	130	85
C03	Female	58	165	68	24.98	110	75
C04	Female	51	170	85	29.41	140	90
C05	male	37	175	95	31.02	135	85
C06	male	50	160	55	21.48	115	75
C07	Female	56	185	85	24.8	125	80
C08	Male	38	170	100	34.6	140	90
C09	Male	60	175	80	26.12	120	80
C10	female	42	160	70	27.34	115	80
C11	Female	30	155	65	27.2	110	75
C12	male	57	160	72	28.13	130	85
C13	Male	35	180	80	24.69	125	85
C14	Female	30	165	70	25.71	115	80
C15	Male	31	175	90	29.39	140	90
C16	Male	61	160	65	25.39	130	85
C17	Male	38	175	85	27.76	130	90
C18	Male	44	180	85	26.23	125	85
C19	Male	65	160	70	27.34	120	80
C20	male	33	175	80	26.12	115	75
C21	Male	39	165	75	27.55	130	85
C22	female	33	155	60	24.97	110	70
C23	Female	69	175	85	27.76	125	80
C24	Female	48	170	80	27.68	130	85
C25	Male	60	160	55	21.48	110	75
C26	Male	33	175	85	27.76	130	85
C27	Male	43	160	70	27.34	120	80
C28	Male	60	175	80	26.12	125	80
C29	Female	32	180	85	26.23	120	80
C30	Male	56	175	85	27.76	125	80

Master chart for biochemical parameters and R219K & C69T genotype of healthy controls

Control Sample ID	HbA1c	Total Cholestrol	TGL	HDL	LDL	VLDL	FBS	ABCA1 Genotype C69T	ABCA1 Genotype R219K
C01	5	215	101.8	49.5	145.14	20.36	90.3	CC	AA
C02	5.7	131	124.6	38.5	67.58	24.92	94.9	CT	GG
C03	5.5	203	125.3	42.3	135.64	25.06	110.7	TT	GA
C04	5.7	201	356.8	40	89.64	71.36	95.8	CT	GA
C05	5.7	130	195.6	40.7	50.18	39.12	84	CC	AA
C06	5.5	159	152.2	40	88.56	30.44	89.9	TT	GA
C07	5.7	238	172.7	40	163.46	34.54	89.7	CC	AA
C08	5.2	130	179.1	28.8	65.38	35.82	86	CT	GG
C09	5.2	150	78.5	36	98.3	15.7	83.6	CC	GG
C10	5.1	115	178.5	48.9	30.4	35.7	95.9	CT	GG
C11	5	201	82.6	44.2	140.28	16.52	85.4	CT	GA
C12	5.5	180	154.2	36	113.16	30.84	86.9	TT	GG
C13	5.1	105	82.4	39.5	49.02	16.48	96.9	CT	GG
C14	5.2	149	89.7	40	91.06	17.94	83.5	CC	GG
C15	5	162	155.6	38	92.88	31.12	78.6	CT	GG
C16	5.7	203	287.4	40	105.52	57.48	85.1	CC	AA
C17	5.3	190	170.3	40	150.63	37.38	95.4	CC	AA
C18	5.3	210	198.6	42	128.28	39.72	102.1	CC	GG
C19	5.1	105	62.6	34.6	57.88	12.52	87.6	CT	GG
C20	5.1	146	209.1	47.6	56.58	41.82	91.9	CC	GA
C21	5.1	198	164.9	38	127.02	32.98	91.9	TT	GA
C22	5.3	140	72	54.1	71.5	14.4	82.9	TT	AA
C23	5	133	148.7	38	65.26	29.74	107.5	CT	GA
C24	5	125	141.8	38	58.64	28.36	103.2	CC	AA
C25	5.6	115	70.9	36	64.82	14.18	94.1	CC	GG
C26	5.2	167	101.2	35	111.76	20.24	85.2	CT	AA
C27	5.7	95	111.3	40	32.74	22.26	94.9	TT	GG
C28	5.2	156	158.3	36	88.34	31.66	116.6	CT	GA
C29	5.3	179	78.7	40.9	122.36	15.74	86.1	CC	GG
C30	5.5	138	85.7	40	80.86	17.14	90.7	CT	GA